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<b>(21) International Application Number:</b> PCT/EP97/00676 <b>(22) International Filing Date:</b> 13 February 1997 (13.02.97)  <b>(30) Priority Data:</b> 08/601,515      14 February 1996 (14.02.96)      US 08/634,995      19 April 1996 (19.04.96)      US  <b>(71) Applicants (for all designated States except US):</b> NOVARTIS AG [CH/CH]; Schwarzwaldallee 215, CH-4058 Basle (CH). NEW ENGLAND DEACONESS HOSPITAL CORPORATION [US/US]; 185 Pilgrim Road, Boston, MA 02215 (US).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> BACH, Fritz, H. [US/US]; 8 Blossom Lane, Manchester-By-The-sea, Boston, MA 01966 (US). FERRAN, Christiane [FR/US]; 165 Winchester Street, Brookline, MA 02146 (US).  <b>(74) Common Representative:</b> NOVARTIS AG; Patent and Trade-mark Division, Lichtstrasse 35, CH-4002 Basle (CH).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.          Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> GENE THERAPY OF ENDOTHELIAL CELLS WITH ANTI-APOPTOTIC PROTEINS FOR TRANSPLANTATION AND INFLAMMATORY CONDITIONS		
<b>(57) Abstract</b> <p>A method of genetically modifying mammalian, especially endothelial cells to render them less susceptible to an inflammatory or other immunological activation stimulus is described, which comprises inserting in that cell or a progenitor thereof DNA encoding an anti-apoptotic polypeptide capable of inhibiting NF-<math>\kappa</math>B and expressing the protein, whereby NF-<math>\kappa</math>B in the cell is substantially inhibited in the presence of a cellular activating stimulus. Suitable polypeptides are selected from those having activity of a mammalian A20, BCL-2, BCL-X<sub>L</sub> (MCL-1) or A1 protein, including homologs and truncated forms of the native proteins. The BCL-2, BCL-X<sub>L</sub> or A1 active polypeptides can also be employed as homodimers or as heterodimers with another anti-apoptotic polypeptide of the BCL family. The method, which can be carried out in vivo or ex vivo or in vitro, is particularly useful in connection with allogeneic or, especially, xenogeneic transplantation, as well as to treat systemic or local inflammatory conditions. Transgenic or somatic recombinant non-human mammals can be prepared expressing such a polypeptide on a regulable basis by the endothelial cells thereof, and tissues or organs comprising such cells can be obtained for grafting into a mammalian recipient.</p>		

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**GENE THERAPY OF ENDOTHELIAL CELLS WITH ANTI-APOPTOTIC PROTEINS FOR TRANSPLANTATION AND INFLAMMATORY CONDITIONS****Field of the invention**

The invention relates to the field of anti-apoptotic gene therapy for transplantation and inflammatory conditions. It provides improvements in the field of gene therapy and tissue and organ transplantation. In its broad aspect, it relates to methods of treating cellular activation processes. In particular, it is concerned with genetic modification of endothelial cells to render them less susceptible to an inflammatory, immunological, or other activating stimulus.

The invention is specifically directed to genetic modification of a cell, in particular an endothelial cell, to render it capable of expressing a polypeptide capable of inhibiting cellular apoptosis, and to recombinant vectors therefor. Examples of polypeptides capable of inhibiting apoptosis in mammalian cells include polypeptides having activity of a mammalian A20 protein, as well as, more generally, polypeptides having anti-apoptotic activity, in particular certain proteins of the BCL family.

The invention also concerns the resultant genetically modified cells, or tissues or organs comprising these cells; and non-human transgenic or somatic recombinant animals so modified.

The invention is most particularly directed to transplantation of genetically modified cells, or graftable tissues or organs comprising such cells, into a mammalian recipient. The mammalian recipient may be allogeneic or xenogeneic as to the cells.

**Background of the invention**

The well-characterized problem of "hyperacute rejection" accompanying transplantation of organs between discordant species, involving an immediate immunological response of recipient antibodies and complement system against the transplanted organ, has been addressed by various means, including the use of immune suppressants, as well as donor organs that express factors which inhibit the complement system of the recipient (Dalmaso, A.P., Immunopharmacology 24 (2) [1992] 149-160).

However, a further condition associated with grafted tissue or organs, and with cells subjected to inflammatory processes in general, is the process known as "activation". In particular, endothelial cell "activation" refers to a continuum of changes characterizing endothelial cells which are subjected to a stimulus such as a cytotoxic cytokine [e.g., tumor necrosis factor (TNF)], an inflammatory or infectious condition, reperfusion injury, atherosclerosis, vasculitis or graft rejection. The endothelium (also referred to as the "vascular endothelium") consists of a layer of cells that line the cavities of the heart and of the blood and lymph vessels. The initial cellular response of such cells to an activating stimulus (often referred to as "Type I" activation) typically involves changes in the cell phenotype, such as retraction of cells from one another, hemorrhage and edema, and trans-migration of leukocytes across the endothelium. A still further phase of cellular activation ("Type II" activation), involves transcriptional up-regulation of various genes encoding interleukins, adhesion molecules, and procoagulant, prothrombotic components of the coagulation system. For example, E-selectin is a tissue specific molecule which is expressed exclusively by endothelial cells (EC) upon activation, and therefore is a generally accepted indicator of Type II EC activation (Pober, J.S. and Cotran, R.S., Transplantation 50 [1990] 537-544).

A recognized phenomenon associated with continuous overexpression of such activation proteins, at the expense of normal cell functioning, is the tendency of the cell to undergo a process of active cellular suicide known as "apoptosis" (G. T. Williams and C. A. Smith, Cell 74 [1993] 777-779; D.L. Vaux et al., Cell 76 [1994] 777-779). Apoptosis can be considered as preprogrammed cell death seen in the process of development, differentiation, or turnover of tissues (Wyllie, A. H. et al., Int. Rev. Cytol. 68 [1980] 251-306). Cell death by apoptosis occurs when a cell activates an internally

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encoded suicide program as a result of either extrinsic or intrinsic signals. Morphologically, apoptosis is characterized by loss of contact with neighboring cells, concentration of cytoplasm, endonuclease activity-associated chromatin condensation and pyknosis, and segmentation of the nucleus, among others. Disappearance of microvilli from the cell surface and vesicle formation on the cell surface (membrane blebbing) are also observed. The remaining fragments of apoptotic body cells are ultimately phagocytosed by neighboring cells (Duvall, E. and Wyllie, A. H., Immunology Today 7(4) [1986] 115-119; Trauth, B.C. et al., Science 245 [1989] 301-305). Apoptotic cell death is of fundamental importance in inflammation, embryogenesis and lymphocyte selection. Avoidance of cell activation and apoptotic cell death accompanying inflammation in general, and particularly in connection with organ transplantation, has become a major goal for workers in the art. Graft injury and loss occurring in connection with graft preservation techniques, as well as accompanying graft rejection, exemplify the vulnerability of endothelial cells to such processes.

An identified transcription factor for many of the genes susceptible to transcriptional up-regulation in response to an activation stimulus such as  $\text{TNF}\alpha$ , is "Nuclear Factor  $\kappa\text{B}$ ", i.e. NF- $\kappa\text{B}$  (M. Grilli et al., International Review of Cytology 143 [1993] 1-61). NF- $\kappa\text{B}$  exists as a preformed transcription factor in the cytoplasm of cells, which is inactivated by its association with a protein inhibitor of the I $\kappa\text{B}$  family. On exposure to cellular activating stimuli such as lipopolysaccharide (LPS), TNF, or oxygen radicals, the I $\kappa\text{B}$  protein is rapidly phosphorylated and then degraded, thereby liberating the preformed NF- $\kappa\text{B}$  and allowing its transmigration to the nucleus. In the nucleus, the binding of NF- $\kappa\text{B}$  to certain NF- $\kappa\text{B}$  binding sites (also referred to as " $\kappa\text{B}$  elements") in promoter regions of the nuclear DNA initiates transcription of genes directly or indirectly under the control of said promoters. Genes subject to up-regulation by NF- $\kappa\text{B}$  upon stimulation of the cell with TNF, include E-selectin, IL-8, and tissue factor, among others (F.H. Bach et al., Immunological Reviews 141 [1994] 1-30; T. Collins, Lab. Invest. 68 [1993] 499-508; M.A. Read et al., J. Exp. Med. 179 [1994] 503-512).

For example, the A20 gene is found to be inducible by TNF or other cellular activating factors (A.W. Opipari et al., J. Biol. Chem. 265 [1990] 14705-14708; C.D. Laherty et al., J. Biol. Chem. 268 [1993] 5032-5039). There is evidence that A20 belongs to a sub-set of TNF-inducible genes which assist in ultimately

conferring resistance to TNF-induced apoptosis (M. Tewari et al., *J. Immunol.* **154** [1995] 1699-1706; A.W. Opipari et al., *J. Biol. Chem.* **267** [1992] 12424-12427; A.W. Opipari et al., *J. Biol. Chem.* **265** [1990] 14705-14708; Dixit et al. [1989], *supra*). A. Krikos and co-workers (*J. Biol. Chem.* **267** [1992] 17971-17976) demonstrated that induction of the A20 gene by TNF $\alpha$  is also mediated by NF- $\kappa$ B binding sites in the A20 promoter (see also C.D. Laherty et al., *J. Biol. Chem.* **268** [1993] 5032-5039).

Besides the A20 protein, certain proteins of the BCL (also referred to as BCL-2) family of proteins also exert an anti-apoptotic effect. Such proteins include BCL-2, BCL-X<sub>L</sub>, MCL-1, and A1. However, the precise mechanisms by which the A20 protein or BCL proteins exert an anti-apoptotic effect have not been completely elucidated.

#### Summary of the invention

An important means of suppressing NF- $\kappa$ B-mediated activation of a cell has now been found. Unexpectedly, it was found that NF- $\kappa$ B regulation of gene transcription is related to expression of an apoptosis inhibiting (i.e. "anti-apoptotic") protein. More particularly, it has been found that such a protein can exert a negative feedback control on NF- $\kappa$ B-mediated gene transcription, namely, the anti-apoptotic protein functions as an inhibitor of the NF- $\kappa$ B transcription factor. This observed negative feedback effect may perhaps in certain cases be exerted via an anti-oxidative mechanism that directly or indirectly protects the NF- $\kappa$ B-I $\kappa$ B complex from dissociating, apparently by acting upstream of I $\kappa$ B degradation. Such inhibitory function may normally assist in preventing apoptotic cell death. However, under conditions of severe cellular challenge, such as occurring in connection with transplantation, and particularly xenotransplantation, expression of the anti-apoptotic protein in a cell may be at insufficient levels, or delayed relative to the rapid activation of NF- $\kappa$ B in the cell, so that inhibition of NF- $\kappa$ B is rendered ineffective to prevent cellular activation and apoptosis.

This finding has now been used to devise a method to treat endothelial or other cells susceptible to an inflammatory or other activating stimulus, and in particular to treat cells, tissues or organs which are subject to transplantation rejection. The method and other aspects of the invention may be used to treat inflammation or disease states

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associated with inflammation, e.g., septic shock, chronic rejection, xenograft rejection, atherosclerosis (restenosis), vasculitis, cardiac failure, or autoimmune diseases.

The invention relies on gene therapy techniques, utilizing an anti-apoptotic gene and its expressed product to inhibit NF- $\kappa$ B activation in mammalian cells susceptible to an activating stimulus.

Accordingly, in a first aspect the invention provides a mammalian cell (in particular, an endothelial cell) which is genetically modified to express an anti-apoptotic protein which is capable of substantially inhibiting NF- $\kappa$ B activation in the presence of a cellular activating stimulus. An example of a "cellular activating stimulus" is tumor necrosis factor, TNF (i.e. TNF $\alpha$ ).

By "NF- $\kappa$ B activation" is meant NF- $\kappa$ B-mediated up-regulation of genes which are directly or indirectly under the control of an NF- $\kappa$ B binding site, such as, e.g., E-selectin in endothelial cells. In functional terms, NF- $\kappa$ B activation constitutes the binding of NF- $\kappa$ B to  $\kappa$ B regulatory sequences in the DNA of a cell in a manner sufficient (whether alone or in combination with other factors) to initiate transcription of a gene in operative association with said sequences.

By "NF- $\kappa$ B inhibition" is meant that NF- $\kappa$ B binding to NF- $\kappa$ B binding sites in the nuclear DNA is prevented. NF- $\kappa$ B is considered "substantially inhibited" when, for example, transcription of the E-selectin gene by an endothelial cell genetically modified according to the invention and stimulated with TNF $\alpha$  is reduced by 60% or greater, and preferably 80% or greater, and even 90% or greater, e.g., 95% and even 99% or greater, relative to an unmodified cell (i.e. a cell not subject to genetic manipulation according to the invention) which is also stimulated by TNF $\alpha$ .

The invention in its broader aspects also concerns a method of genetically modifying mammalian (e.g., endothelial) cells to render them less susceptible to an inflammatory or other immunological activation stimulus by inserting in these cells, or progenitors thereof, DNA encoding an anti-apoptotic protein capable of inhibiting NF- $\kappa$ B and expressing the protein, whereby NF- $\kappa$ B in the cell is substantially inhibited in the presence of a cellular activating stimulus.

It was found that inhibition of NF- $\kappa$ B-initiated transcription by the anti-apoptotic protein, such as, e.g., an A20 protein, in a genetically modified cell is unexpectedly potent, even at moderate levels of transfection in vitro with the corresponding A20 gene (e.g., 0.5  $\mu$ g plasmid DNA per approximately  $5 \times 10^5$  cells), leading to effective suppression of induction of cytokine-inducible genes such as tissue factor, E-selectin and IkB $\alpha$ , all of which are associated with inflammation.

It will be apparent that such a therapy will be useful in general to treat patients afflicted with conditions which may benefit from inhibition of NF- $\kappa$ B activation, such as inflammation. Such a therapy will also be useful to moderate complications occurring in connection with organ transplantation, especially where the graft recipient is human, and most particularly where the graft is xenogeneic as to the recipient.

Thus in a further aspect, the invention comprises a method of transplanting donor endothelial or other mammalian cells (e.g., bone marrow stem cells as precursors of monocytes, NK cells, or lymphocytes; or islet cells), or graftable tissues or organs comprising such cells, to a mammalian recipient in whose blood or plasma these cells, tissues or organs are subject to activation, which comprises:

- (a) genetically modifying the donor cells, or progenitor cells thereof, by inserting therein DNA encoding an anti-apoptotic protein capable of inhibiting NF- $\kappa$ B, and
- (b) transplanting the resultant modified donor cells, or tissues or organs comprising these cells, into the recipient, and expressing in the cells the anti-apoptotic protein, whereby NF- $\kappa$ B activation in the cells is substantially inhibited in the presence of a cellular activating stimulus.

The "modified donor cells" of step (b) will be understood to refer to cells which themselves are subjected to genetic modification in step (a), as well as to progeny thereof.

According to a further aspect of the invention, there are provided donor endothelial cells, and tissues and organs comprising such cells, wherein the cells are genetically modified to regulably or constitutively express an anti-apoptotic protein in a graft recipient, whereby NF- $\kappa$ B is substantially inhibited, for transplantation into a recipient species. The graft recipient may be allogeneic or xenogeneic as to the donor cells, tissues or organs. In its additional aspects, the invention provides a non-human



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transgenic or somatic recombinant mammal comprising DNA encoding an anti-apoptotic protein of a different species; and a method of preparing such non-human transgenic or somatic recombinant mammal. Also within the scope of the invention are vectors for genetically modifying cells by insertion of anti-apoptotic protein-encoding polynucleotides, such as for example retroviral vectors, and especially, adenoviral vectors.

**Description of the drawings**

**Figure 1:** Analysis of antibody affinity purified protein extracted from: BAEC transfected with A20 vector ("A20"), BAEC transfected with empty pAC vector ("PAC"), or non-transfected BAEC ("NT") following stimulation with TNF $\alpha$ . Also analyzed for comparison is HUVEC which is either non-stimulated ("NS") or stimulated with TNF $\alpha$  ("TNF").

**Figure 2:** Luciferase levels in relative light units (RLU) in BAEC co-transfected with A20 and/or pAC vector ("pAC") together with the porcine E-selectin promoter region cloned into a luciferase expressing vector ("porcine E-selectin Reporter"); BAEC are either non-stimulated ("NS" or "control") or stimulated with TNF $\alpha$  ("TNF") or lipopolysaccharide ("LPS").

**Figures 3A-3C:** Luciferase levels in BAEC co-transfected with either A20 or pAC and one of the following promoters cloned into a luciferase vector: (a) human IL-8 promoter ("IL-8 Reporter") (FIG. 3A); (b) porcine I $\kappa$ B $\alpha$  promoter ("I $\kappa$ B $\alpha$  Reporter") (FIG. 3B); and (c) porcine tissue factor (TF) promoter ("Tissue Factor Reporter") (FIG. 3C); and then stimulated with TNF $\alpha$  or LPS or maintained as a control.

**Figure 4:** Luciferase levels in BAEC co-transfected with either A20 or pAC and  $\kappa$ B elements derived from the porcine E-selectin promoter cloned into a luciferase vector ("NF $\kappa$ B Reporter"), and then stimulated with TNF $\alpha$  or LPS or maintained as a control.

**Figure 5A:** Luciferase levels in BAEC co-transfected with either A20 or pAC and an RSV-LTR driven luciferase vector ("RSV-LUC Reporter").

**Figure 5B:** <sup>14</sup>C-labeled chloramphenicol levels, in counts per minute (CPM), in BAEC co-transfected with A20 and/or pAC and an HIV LTR-driven CAT vector ("HIV-CAT Reporter"). Cells are stimulated with the viral c-Tat protein ("C-Tat") or maintained as a control.

**Figures 6A, 6B, 6C:** Luciferase levels in BAEC co-transfected with pAC and either Bcl-2 or Bcl-X<sub>L</sub>, together with either the E-selectin reporter (FIG. 6a), the I $\kappa$ B $\alpha$  reporter (FIG. 6B), or the NF  $\kappa$ B reporter (FIG. 6C) cloned into a luciferase vector, and then stimulated with TNF or LPS or maintained as a non-stimulated control.

**Figure 7:** Luciferase levels in BAEC co-transfected with pAC, full length A20, or truncated A20 clones #3 ["tA20(3)"] or #7 ["tA20(7)"], together with the E-selectin reporter cloned into a luciferase vector, and then stimulated with TNF or LPS or maintained as a non-stimulated ("NT") control.

**Figure 8:** EMSA of nuclear extracts from TNF-stimulated (+) or non-stimulated (-) PAEC infected with adenoviral Bcl-2 ("rAd.Bcl-2") or, as a control,  $\beta$ -gal ("rAd. $\beta$ -Gal"), using a  $\kappa$ B binding oligonucleotide derived from the human immunoglobulin (Ig)  $\kappa$  promoter and, for comparison, a cold wild-type NF $\kappa$ B-specific probe ("sp-comp.") and a non-specific competitor ("nsp. comp.")(AP-1).

**Figure 9:** Western blot of rAd.Bcl-2- (or, as a control, rAd. $\beta$ -gal-) infected PAEC taken prior to ("0") , or ten minutes ("10' ") or one hundred-twenty minutes ("120' ") following stimulation with TNF, with I $\kappa$ B $\alpha$  as shown.

**Figure 10:** EMSA of nuclear extracts from rAd.Bcl-2- (or, as a control, rAd. $\beta$ -gal-) infected PAEC prior to ("-") or two hours following (+) TNF stimulation, using the transcription factor cAMP responsive element ("CRE") as a probe and, for comparison, a cold wild-type CRE-specific probe ("sp-comp.") and a non-specific competitor ("nsp. comp.").

**Figure 11:** Luciferase levels in BAEC co-transfected with either A1 or pAC and a luciferase vector comprising 0.7 $\mu$ g of either the (A) E-selectin or (B) NF $\kappa$ B reporter. Cells are stimulated with TNF or LPS or non-stimulated (control).

**Figure 12:** Northern blot TNF-stimulated (+) or non-stimulated (-) HUVEC infected with adenoviral I $\kappa$ B $\alpha$  ("rAd.I $\kappa$ B $\alpha$ ") or A20 ("rAd.A20") or, as a control, rAd. $\beta$ -gal .

**Definitions**

"Graft," "transplant" or "implant" are used interchangeably to refer to biological material derived from a donor for transplantation into a recipient, and to the act of placing such biological material in the recipient.

"Host or "recipient" refers to the body of the patient in whom donor biological material is grafted.

"Allogeneic" refers to the donor and recipient being of the same species (also "allograft").

As a subset thereof, "syngeneic" refers to the condition wherein donor and recipient are genetically identical. "Autologous" refers to donor and recipient being the same individual. "Xenogeneic" (and "xenograft") refer to the condition where the graft donor and recipient are of different species.

"A20" refers to a natural mammalian A20 gene (including the cDNA thereof) or protein, including derivatives thereof having variations in DNA (or amino acid) sequence (such as silent mutations or deletions of up to 5 amino acids) which do not prejudice the capability of the natural protein to block NF- $\kappa$ B activation. The A20 gene (protein) may, for example, be porcine, bovine or human, or may be of a primate other than human, depending on the nature of the cells to be modified and the intended recipient species for transplantation.

"A polypeptide having activity of an A20 protein" or "A20 active protein" refers to a protein which is able to block or suppress NF- $\kappa$ B activation, and which is at least 70%, preferably at least 80%, and more preferably at least 90% (most preferably at least 95%) homologous to the protein sequence of a natural mammalian (e.g., human) A20 protein (for example, SEQ. ID. NO. 1 hereof). In a preferred embodiment, the A20 protein of the invention is human and has the amino acid sequence corresponding to SEQ. ID. NO. 1 herein (as disclosed in A.J. Opipari et al. [1990], *supra*). In a further aspect, the A20 gene of the invention is at least 70%, and more preferably at least 80%, or at least 90% (e.g., at least 95%) homologous to, or corresponds to, SEQ. ID. NO. 2 herein.

"Bcl-2" refers to a natural mammalian Bcl-2 gene (including the cDNA thereof) or protein (denoted by capital letters), including derivatives thereof having variations in DNA (or amino acid) sequence (such as silent mutations or deletions of up to 5 amino acids) which do not prejudice the capability of the natural protein to block NF- $\kappa$ B activation. The Bcl-2 gene (protein) may, for example, be porcine, bovine or human, or may be of a primate other than human, depending on the nature of the cells to be modified and the intended recipient species for transplantation.

"A polypeptide having activity of BCL-2 protein" or "BCL-2 active protein" refers to a protein which is able to block or suppress NF- $\kappa$ B activation, and which is at least 70%, preferably at least 80%, and more preferably at least 90% (most preferably at least 95%) homologous to the protein sequence of a natural mammalian (e.g., human) BCL-2 (for example, SEQ. ID. NO. 3 hereof). In a preferred embodiment of the invention, the BCL-2 polypeptide of the invention is human and has the amino acid sequence corresponding to SEQ. ID. NO. 3 (as disclosed by Tsujimoto, Y. and Croce, C.M., PNAS **83** [1986] 5214-5218, and in WO 95/00642).

Similarly, "Bcl-x<sub>L</sub>" refers to a natural mammalian Bcl-x<sub>L</sub> gene (including the cDNA thereof) or protein (denoted by capital letters), including derivatives thereof having variations in DNA (or amino acid) sequence (such as silent mutations or deletions of up to 5 amino acids) which do not prejudice the capability of the natural protein to block NF- $\kappa$ B activation. The Bcl-x<sub>L</sub> gene (protein) may, for example, be porcine, bovine or human, or may be of a primate other than human, depending on the nature of the cells to be modified and the intended recipient species for transplantation.

"A polypeptide having activity of BCL-X<sub>L</sub> protein" or "BCL-X<sub>L</sub> active protein" refers to a protein which is able to block or suppress NF- $\kappa$ B activation, and which is at least 70%, preferably at least 80%, and more preferably at least 90% (most preferably at least 95%) homologous to the protein sequence of a natural mammalian (e.g., human) BCL-X<sub>L</sub> protein (for example, SEQ. ID. NO. 4 hereof). In a preferred embodiment of the invention, the BCL-X<sub>L</sub> polypeptide of the invention is

human and has the amino acid sequence corresponding to SEQ. ID. NO. 4 (as also disclosed in WO 95/00642).

"A1" refers to a natural mammalian A1 gene (including the cDNA thereof) or protein, including derivatives thereof having variations in DNA (or amino acid) sequence (such as silent mutations or deletions of up to 5 amino acids) which do not prejudice the capability of the natural protein to block NF- $\kappa$ B activation. The A1 gene (protein) employed in the invention may, for example, be porcine, bovine or human, or may be of a primate other than human, depending on the nature of the cells to be modified and the intended recipient species for transplantation.

"A polypeptide having activity of A1 protein" or "A1-active protein" refers to a protein which is able to block or suppress NF- $\kappa$ B activation, and which is at least 70%, preferably at least 80%, and more preferably at least 90% (most preferably at least 95%) homologous to the protein sequence of a natural mammalian (e.g., human) A1 (for example, SEQ. ID. NO. 5 hereof). In a preferred embodiment of the invention, the A1 polypeptide of the invention is human and has the amino acid sequence corresponding to SEQ. ID. NO. 5 (as disclosed in A. Karsan et al., Blood, 87, No. 8 [April 15, 1996] 3089-3096).

**Detailed description**

The human A20 gene was originally cloned as an immediate early response gene which is rapidly but transiently expressed following TNF treatment of human umbilical vein endothelial cells (HUVEC) (Opipari et al. [1990], supra). It is now known that a protein having A20 activity can also be induced by other stimuli such as IL-1 in HUVEC (Dixit et al. [1989], supra); CD40 cross-linking in B cells (Tewari et al. [1995], supra); or phorbol 12-myristate 13-acetate (PMA) or HTLV-I Tax protein in Jurkat T cells (Laherty et al. [1993], supra). An A20 protein is also constitutively present in mature resting T cells.

A cDNA sequence of the human A20 gene obtained from HUVEC, and the deduced amino acid sequence, are published by Opipari et al. [1990], supra, as indicated hereinabove. TNF-induction of A20 has been indicated to be mediated through NF- $\kappa$ B binding sites in the A20 promoter, extending from -45 to -54 (5'-GGAAATCCCC-3') and from -57 to -66 (5'-GGAAAGTCCC-3') of the gene. At the protein level, the deduced sequence of 790 amino acids (SEQ. ID. NO. 1) contains within its carboxyl terminal half 7 Cys/Cys<sub>2</sub> zinc finger repeats: six with the configuration Cys-X<sub>4</sub>-Cys-X<sub>11</sub>-Cys-X<sub>2</sub>-Cys and one with the configuration Cys-X<sub>2</sub>-Cys-X<sub>11</sub>-Cys-X<sub>2</sub>-Cys, wherein X is any amino acid and the subscripts represent numbers of amino acids between each of the indicated cysteines. A novel finger loop domain composed of 11 amino acid residues has also been identified (Krikos et al. [1992], supra).

In one embodiment of this invention, the "protein having A20 activity" comprises amino acid residues 386-790 of SEQ. ID. NO. 1, comprising the zinc finger region of the native protein sequence (i.e. having 7 zinc binding domains), or a region at least 80% homologous to said residues. Another suitable truncated form of the native human protein consists essentially of residues 373-790 of SEQ. ID. NO. 1 hereof. Other deletion mutants found to be capable of inhibiting NF $\kappa$ B comprise the N-terminus and 2 zinc-binding domains of the polypeptide, e.g., amino acids 1-538 of SEQ. ID. NO. 1.

It has been found that the A20 protein acts with specificity to inhibit NF $\kappa$ B. For example, expression of JunB, another TNF or LPS-inducible protein, is not found to be inhibited by A20 expression under conditions in which NF $\kappa$ B is so inhibited.

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The *bcl-2* gene was originally cloned from the breakpoint of a t(14;18) translocation present in many human B cell lymphomas. In vitro, BCL-2 protein has been shown to prevent apoptotic cell death selectively in certain cell lines, suggesting the existence of multiple independent intracellular mechanisms of apoptosis, some of which can be prevented by BCL-2 and others of which are apparently unaffected by the gene (WO 95/00642). Native proteins of the BCL (i.e. BCL-2) family are characterized by three conserved regions, referred to as BCL-2 homology regions 1, 2 and 3 (abbreviated as BH-1, BH-2 and BH-3), that are required for regulation of apoptosis and protein-protein interaction. Proteins of the BCL family include anti-apoptotic polypeptides such as BCL-2, BCL-X<sub>L</sub> (the long form of a splice variant of BCL-X), MCL-1 and BAG-1.

Another member of the BCL family comprises the A1 protein. Human A1 has been found to comprise the BH1 and BH2 regions characteristic of the BCL family (A. Korsan et al., Blood 87, No.8 [April 15, 1996] 3089-3096; A. Korsan et al., J. Biol. Chem. 271 (44) [November 1, 1996] 27201-27204). Suitable anti-apoptotic polypeptides for use in the invention may comprise or consist essentially of regions BH1 and BH2 of native (e.g., human) A1 protein, or an amino acid sequence which in the aggregate is at least 80%, preferably at least 90%, and more preferably at least 95%, homologous to the aggregate of the BH1 and BH2 regions of the native A1 protein.

In general, suitable deletion mutants of the BCL family may comprise, for example, at least one of the BH1, BH2, BH3 and BH4 regions of the native protein, for example, for each protein, one or more of the following peptide sequences (a.a. = amino acid position no.):

**BCL-2:** about a.a. 10 to about a.a. 30; about a.a. 93 to about a.a. 107; about a.a. 135 to about a.a. 155; about a.a. 187 to about a.a. 202, of SEQ. ID. NO. 3;

**BCL-X<sub>L</sub>:** about a.a. 5 to about a.a. 24; about a.a. 86 to about a.a. 100; about a.a. 129 to about a.a. 148; about a.a. 180 to about a.a. 195, of SEQ. ID. NO. 4;

**A1:** about a.a. 27 to about a.a. 45; about a.a. 66 to about a.a. 99; about a.a. 133 to about a.a. 145, of SEQ. ID. NO. 5.



Still other BCL family apoptosis-regulating polypeptides useful in the invention may comprise CDN-1 and CDN-2 (W0 95/15084); MCL-1 (Yang et al.,

J. Cell. Phys. 166 [1996] 523-536, particularly a polypeptide comprising one or more of amino acid residues 6-25, 209-223, 252-272, and 304-319 thereof; and BAG-1 (or homo- or heterodimers thereof with BCL-2 or other BCL family members) (Takayama et al., Cell, 80 [1995] 279-284).

These anti-apoptotic polypeptides may exist in vivo in the form of homodimers or heterodimers with another anti-apoptotic polypeptide of the BCL family. Such anti-apoptotic polypeptides may also be found in heterodimer combinations with antagonist polypeptides of the BCL family such as BCL-X<sub>s</sub> (the alternatively spliced short form of BCL-X), BAX and BAD.

The present invention also comprises a method of treating the dysfunctional or activation response of a cell to an inflammatory or other activation stimulus, comprising modifying said cell by inserting therein DNA encoding an anti-apoptotic protein, in operative association with a suitable promoter, and expressing said anti-apoptotic protein at effective levels whereby NF- $\kappa$ B activation in said cell is substantially inhibited.

In a particular aspect, the invention comprises a method of treating the dysfunctional or activation response of a cell to an inflammatory or other activation stimulus, comprising modifying the cell by inserting therein DNA encoding a polypeptide having anti-apoptotic activity of an A20 protein in operative association with a suitable promoter, and expressing the polypeptide at effective levels whereby activation in the cell is substantially inhibited.

It further comprises a method of inhibiting cellular activation in a mammalian subject susceptible to an inflammatory or immunological stimulus which comprises genetically modifying endothelial cells of the subject, by insertion of DNA encoding an anti-apoptotic protein capable of inhibiting NF- $\kappa$ B and expressing that protein, whereby NF- $\kappa$ B is substantially inhibited in the cells in the presence of a cellular activating stimulus.

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In a further aspect, it comprises a method of treating the activation response of a cell to an inflammatory or other stimulus, comprising modifying that cell by inserting therein DNA encoding a polypeptide having anti-apoptotic activity of a BCL protein (such as BCL-2 and BCL-X<sub>L</sub> proteins), a homodimer of such a polypeptide, or a heterodimer of such a polypeptide with another anti-apoptotic protein of the BCL family, and expressing the polypeptide or dimer at effective levels whereby activation in the cell is substantially inhibited.

The invention also includes the cells so modified, and corresponding tissues or organs comprising such cells.

The protein-encoding region and/or the promoter region of the inserted DNA may be heterologous, i.e. non-native to the cell. Alternatively, one or both of the protein encoding regions and the promoter region may be native to the cell, provided that the promoter is other than the promoter which normally controls anti-apoptotic (e.g., A20) expression in the cell. The protein coding sequence may be under the control of an appropriate signal sequence, e.g., a nucleus specific signal sequence.

Preferably the protein encoding region is under the control of a constitutive or regulable promoter. By "constitutive" is meant substantially continuous transcription of the gene and expression of the protein over the life of the cell. By "regulable" is meant that transcription of the gene and expression of the protein is related to the presence, or absence, of a given substance. An embodiment of "regulable" expression comprises "inducible" expression, i.e. whereby transcription (and thus protein expression) occurs on demand in response to a stimulus. The stimulus may comprise endothelial cell activating stimuli or a predetermined external stimulus. The endothelial cell activating stimuli may be any of the stimuli which give rise to changes in the endothelium of donor tissue or organs which stimulate coagulation. The predetermined external stimulus may be a drug, cytokine or other agent.

An advantage of employing an inducible promoter for transplantation purposes is that the desired high level expression of the (e.g., A20) active protein can be obtained on demand in response to a predetermined stimulus, such as e.g., the presence of tetracycline in the cellular environment. An example of a tetracycline-inducible promoter which is suitable for use in the invention is disclosed in P.A. Furth et al., PNAS 91 [1994] 9302-9306. Alternatively, an example of a regulable promoter system in which

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transcription is initiated by the withdrawal of tetracycline is described in M. Gossen and H. Bujard, PNAS 89 [1992] 5547-5551.

Preferably, expression of the (e.g., A20) active protein is induced in response to a predetermined external stimulus, and the stimulus is applied beginning immediately prior to subjecting the cells to an activating stimulus, so that expression is already at effective levels to block NF- $\kappa$ B activation. For example, cells of a donor mammal (e.g., porcine) may be genetically modified according to the invention by insertion of an anti-apoptotic gene (e.g., porcine or human) under the control of a promoter which is inducible by a drug such as tetracycline. The animal, whether somatic recombinant or transgenic, may be raised up to the desired level of maturity under tetracycline-free conditions, until such time as the cells, or tissue or organs comprising the cells, are to be surgically removed for transplantation purposes. In such case, prior to surgical removal of the organ, the donor animal may be administered tetracycline in order to begin inducing high levels of expression of the anti-apoptotic (e.g., A20) protein. The organ can then be transplanted into a recipient (e.g., human), and tetracycline may continue to be administered to the recipient for a sufficient time to maintain the protein at the desired levels in the transplanted cells to inhibit NF- $\kappa$ B activation. Alternatively, after being surgically removed from the donor, the organ can be maintained ex vivo in a tetracycline-containing medium until such time as grafting into a recipient is appropriate.

In another embodiment, expression may be provided to occur as a result of withholding tetracycline from the cellular environment. Thus, cells of a donor animal may be genetically modified according to the invention by insertion of a gene encoding an anti-apoptotic (e.g., A20) protein under the control of a promoter which is blocked by tetracycline, and which is induced in the absence of tetracycline. In such case, the animal may be raised up to the desired level of maturity while being administered tetracycline, until such time as the cells, tissues or organs of the animal are to be harvested. Prior to surgical removal, the donor animal may be deprived of tetracycline in order to begin inducing expression of the protein, and the patient in whom the cells, tissue or organs are transplanted may thereafter also be maintained tetracycline-free for a sufficient time to maintain appropriate levels of expression.

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Preferably, the inserted DNA sequences are incorporated into the genome of the cell. Alternatively, the inserted sequences may be maintained in the cell extrachromosomally, either stably or for a limited period.

The modification of endothelial or other mammalian cells according to the invention may be carried out *in vivo* or *ex vivo*.

Thus the invention also comprises a method for inhibiting the dysfunctional or activation response of endothelial cells to an inflammatory or other activation stimulus *in vivo* in a patient in need of such therapy, comprising modifying such cells of the patient by inserting in the cells DNA encoding an anti-apoptotic protein in operative association with a constitutive or inducible promoter and expressing the protein at effective levels whereby NF- $\kappa$ B activation is substantially inhibited. For example, the blood vessels of an organ (e.g., a kidney) can be temporarily clamped off from the blood circulation of the patient and the vessels perfused with a solution comprising a transmissible vector construct containing the anti-apoptotic (e.g., A20) gene, for a time sufficient for at least some cells of the organ to be genetically modified by insertion therein of the vector construct; and on removal of the clamps, blood flow can then be restored to the organ and its normal functioning resumed.

In another aspect, cell populations can be removed from the patient or a donor animal, genetically modified *ex vivo* by insertion of vector DNA, and then re-implanted into the patient or transplanted into another recipient. For example, an organ can be removed from a patient or donor, subjected *ex vivo* to the perfusion step described above, and the organ can be re-grafted into the patient or implanted into a different recipient of the same or different species.

For gene delivery, retroviral vectors, and in particular replication-defective retroviral vectors lacking one or more of the gag, pol, and env sequences required for retroviral replication, are well-known in the art and may be used to transform endothelial or other mammalian cells. PA317 or other producer cell lines producing helper-free viral vectors are well-described in the literature (A.D.Miller and C.Buttimore, Mol. Cell. Biology 6 [1986] 2895-2902). A representative retroviral construct comprises at least one viral long terminal repeat and promoter sequences upstream of the

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nucleotide sequence of the therapeutic substance and at least one viral long terminal repeat and polyadenylation signal downstream of the nucleotide sequence.

Vectors derived from adenoviruses, i.e. viruses causing upper respiratory tract disease and also present in latent infections in primates, are also known in the art. The ability of adenoviruses to attach to cells at low ambient temperatures is an advantage in the transplant setting which can facilitate gene transfer during cold preservation of tissue or organs. Adenoviral-mediated gene transfer into vessels or organs by means of transduction perfusion as described hereinabove is also a means of genetically modifying cells *in vivo* or *ex vivo*.

Alternative means of targeted gene delivery comprise DNA-protein conjugates, liposomes, etc.

In yet another embodiment, the invention comprises a method for suppressing the activation response of donor cells, or tissue or organs comprising such cells, upon transplantation into a mammalian recipient in whom the cells are susceptible to activation, which comprises:

- (a) modifying the donor cells by introducing therein DNA encoding an anti-apoptotic protein; and
- (b) transplanting the resultant donor cells, or tissue or organs comprising such cells, into the recipient and expressing the protein, whereby NF- $\kappa$ B activation of the cells is substantially inhibited.

The donor species may be any mammalian species which is the same or different from the recipient species, and which is able to provide the appropriate cells, tissue or organs for transplantation into the recipient species.

The donor may be of a species which is allogeneic or xenogeneic to that of the recipient. The recipient is a mammal, e.g., a primate, and is preferably human. For human recipients, it is envisaged that human (i.e. allogeneic) as well as pig (i.e. xenogeneic) donors will be suitable, but any other mammalian species (e.g., bovine or non-human primate) may also be suitable as donor.

For example, porcine aortic endothelial cells (PAEC), or the progenitor cells thereof, can be genetically modified to express porcine or human anti-apoptotic, e.g. A20 protein at effective levels, for grafting into a human recipient. Heterologous DNA

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encoding the A20 or other anti-apoptotic protein can be inserted into the animal or an ancestor of the animal at the single-cell or early morula stage. The preferred stage is the single-cell stage, although the process may be carried out between the two and eight cell stages. A transgenic non-human animal can be thereby obtained which will pass the heterologous DNA on to offspring. In another aspect genes can be inserted into somatic/body cells of the donor animal to provide a somatic recombinant animal, from whom the DNA construct is not capable of being passed on to offspring (see, e.g., Miller, A.D. and Rosman, G.J., Biotechniques 7 [1989] 980-990).

Appropriate well-known methods of inserting foreign cells or DNA into animal tissue include micro-injection, embryonic stem cell manipulation, electroporation, cell gun, transduction, transfection, retroviral infection, adenoviruses, etc. In one embodiment, the gene is inserted in a particular locus, e.g., the thrombomodulin locus. Subsequently, the construct is introduced into embryonic stem cells, and the resulting progeny express the construct in a tissue specific manner, paralleling the expression of thrombomodulin, i.e. in the vascular endothelium.

Methods of preparing transgenic pigs are disclosed in e.g. Pinckert et al., Xeno 2, No. 1 [1994] 10-15.

Genetically modified endothelial cells may be administered by intravenous or intra-arterial injection under defined conditions. Tissues or organs comprised thereof may also be removed from a donor and grafted into a recipient by well-known surgical procedures. Prior to implantation, the treated endothelial cells, tissue or organ may be screened for genetically modified cells containing and expressing the construct. For this purpose, the vector construct can also be provided with a second nucleotide sequence encoding an expression product that confers resistance to a selectable marker substance. Suitable selection markers for screening include the neo gene, conferring resistance to neomycin or the neomycin analog, G418.

Although any mammalian cell can be targeted for insertion of the anti-apoptotic gene, such as monocytes, NK cells, lymphocytes, or islet cells, the preferred cells for manipulation are endothelial cells. The recipient species will primarily be human, but other mammals, such as non-human primates, may be suitable recipients.

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In an alternative embodiment of the invention, the anti-apoptotic polypeptide, in a pharmaceutically acceptable carrier, may be applied directly to cells, tissues or organs in vivo.

It will be appreciated that the modified donor cells and tissues and organs defined above have a supplementary function in the prevention of xenotransplant rejection since complement-mediated events also participate in hyperacute rejection of such transplants (A.P. Dalmaso et al., Transplantation 52 [1991] 530-533). Therefore, the genetic material of the cells of the donor organ is typically also altered such that activation of the complement pathway in the recipient is prevented. This may be done by providing transgenic animals that express the complement inhibitory factors of the recipient species. The endothelial cells of a donor organ obtained from such an animal can be modified by gene therapy techniques to provide the endothelial cells defined above. Alternatively a vector containing DNA encoding a protein having anti-apoptotic (e.g., A20) activity can be introduced into the transgenic animal at the single cell or early morula stage. In this way, the resulting transgenic animal will express the complement inhibitory factors and will have endothelial cells as defined above.

Thus in a further aspect the invention also provides endothelial cells, tissue, donor organs and non-human transgenic or somatic recombinant animals as defined above which express one or more human complement inhibitory factors.

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The following Examples are intended to be illustrative only and not limitative of the invention. Cultured BAEC are transfected with reporter constructs consisting of promoters of genes known to be upregulated upon EC activation, i.e. E-selectin, I $\kappa$ B $\alpha$ , IL-8 and tissue factor.

## **EXAMPLES**

### **Materials and methods:**

The following vectors are utilized in the Examples:

"pAC": 8.8 kB plasmid vector containing a CMV promoter, a pUC19 polylinker site, and an SV40 splice/polyA site (J.Herz and R.D.Gerard, *PNAS* **90** [1993] 2812-2816).

A20 expression plasmid ("A20" in Figures): human A20 cDNA (Opipari et al. [1990], *supra*) (SEQ. ID. NO. 2), subcloned into the pAC expression vector at the XbaI restriction site.

Bcl-2 and Bcl-x<sub>L</sub> expression plasmids: murine bcl-2 and bcl-x<sub>L</sub> genes (W. Fang et al., *J. Immunol.* **155** [1995] 66-75). The 830 bp full-length bcl-2 cDNA was flag-tagged and cloned in the PAW neo-3 expression vector into a ClaI/XbaI expression vector. The 700 bp full-length Bcl-x<sub>L</sub> cDNA was also flag-tagged and cloned into a ClaI/BamHI sites of the PAW neo-3 expression vector (PAW neo-3 is a 7kb expression plasmid containing ampicillin and neomycin resistance sites and a SFFV-LTR promoter before the polylinker cloning site) (SFFV = spleen focus forming virus).

Porcine E-selectin reporter: bp -1286 to +484 of the porcine E-selectin promoter cloned into the pMAMneo-luc plasmid vector by replacing the mm TV promoter (Clontech, Palo Alto, CA) (this includes the first complete intron and exon, as well as the beginning of the 2nd exon up to the ATG site).

Porcine NF- $\kappa$ B reporter: 4 copies of NF- $\kappa$ B binding sites derived from the porcine E-selectin promoter inserted upstream of a TK minimal promoter driving the full length luciferase gene in a pT3/T7-luc vector (Clontech).



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The vector backbone is a Bluescript KS+ plasmid (Stratagene, La Jolla CA, USA).

Human IL-8 reporter: human IL-8 (hIL-8) promoter cloned into p-UBT luc.

Porcine TF reporter: -4000 to +34 fragment of the porcine TF promoter cloned into p-UBT luc, a luciferase reporter gene vector (R. de Martin et al., Gene **124** [1993] 137-138), according to the method of T. Moll et al., J. Biol. Chem. **270** [1995] 3849-3857.

Porcine I $\kappa$ B $\alpha$  (also referred to as "ECI-6") reporter: 600 bp fragment of the porcine ECI-6/I $\kappa$ B $\alpha$  promoter ligated into p-UBT-luc, with the creation of an additional Hind III site, as described by R. de Martin et al., EMBO J. **12** [1993] 2773-2779.

HIV-CAT reporter: -117 bp to the TATA box start of the HIV-wt LTR, cloned upstream of the CAT gene (CAT3N polylinker), prepared as described by K. Zimmermann et al., Virology **182** [1991] 874-878.

RSV  $\beta$ -gal reporter: E. coli  $\beta$ -gal gene inserted into the pRc/RSV vector (Invitrogen, San Diego, CA, USA) at the Not I site.

RSV-LUC reporter: full-length luciferase gene cloned into the pRc/RSV vector.

#### Assays:

Cell extracts are assayed for luciferase (or CAT) and galactosidase levels.

a) Luciferase levels (E-selectin, NF- $\kappa$ B, IL-8, TF and I $\kappa$ B $\alpha$  [ECI-6] promoters):

10  $\mu$ l of cellular extract are added to 90  $\mu$ l of a solution containing 24 mM glycylglycine (pH 7.8), 2 mM ATP (pH 7.5) and 10 mM MgSO<sub>4</sub>. Samples are read on a Microlumet LB 96P luminometer (EG+G Berthold) using an injection mix consisting of 24 mM glycylglycine and 0.1 mM luciferin (Boehringer, Mannheim, Germany).

Luciferase activity is normalized for  $\beta$ -galactosidase using the following formula: (luciferase activity/ $\beta$ -gal activity) x 1000. Luciferase activity is also corrected for protein by dividing the luciferase activity by protein concentration. Normalized luciferase activity is given in relative light units (RLU).

CAT levels (HIV LTR activity):

A Promega kit (Promega, Madison, WI, USA) is used to incubate cells in <sup>14</sup>C-labeled chloramphenicol and n-butyryl coenzyme A - containing medium (the CAT protein transfers the n-butyryl moiety of the coenzyme to chloramphenicol). Cells are extracted into xylene, which is mixed with scintillation liquid and counted in a scintillation counter (1900 TR, Packard, Downes Grove, IL, USA). Counts per minute

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(CPM) are normalized for  $\beta$ -galactosidase using the following formula:  
(cpm/ $\beta$ -gal activity) x 1000. Significance is determined by Student's t-test.

**c)  $\beta$ -galactosidase levels:**

The RSV  $\beta$ -gal reporter serves as a control for transfection efficiency. The Tropix, Inc. Galacto-Light protocol (Tropix Inc., Bedford, MA, USA) is employed to measure  $\beta$ -galactosidase levels.

**Example 1: Transfected BAEC express human A20 protein**

Bovine aortic endothelial cells (BAEC) are isolated and cultured in 10 cm plates in Dulbecco's Modified Eagle Medium (DMEM), supplemented with L-glutamine (2 mM), penicillin G (100 units/ml), and fetal calf serum (FCS) (10%). Cells are maintained at 37°C in a humidified incubator with a 5% CO<sub>2</sub> atmosphere. When the cells reach 70% confluency, one group (i.e. approximately  $1 \times 10^6$  cells) is transfected with 0.5  $\mu$ g of the A20 vector ("A20"); a second group is transfected with 0.5  $\mu$ g of the pAC vector ("PAC"); and a third group is maintained as a non-transfected ("NT") control. All transfections are done with 16  $\mu$ g lipofectamine. Non-transfected, non-stimulated HUVEC ("NS") or non-transfected, TNF $\alpha$ -stimulated HUVEC ("TNF") also serves as controls.

Cells are washed twice with cysteine and methionine-free medium (ICN, Lisle, IL, USA), and then placed in the same medium supplemented with 100  $\mu$ Ci/ml Tran <sup>35</sup>S labelled cysteine and methionine (ICN). After four hours, cells are harvested. Immuno-precipitation with polyclonal rabbit anti-human A20 polyclonal serum on a polyacrylamide SDS gel, as shown in FIG. 1, reveals the presence of a <sup>35</sup>S-labelled 80 kD A20 protein in the "A20" extract, but not the "PAC", "NT" or "NS" extracts. This protein is comparable to that seen in the TNF $\alpha$  stimulated HUVEC extract ("TNF").

**Examples 2-4: General Procedure**

Approximately  $3 \times 10^5$  BAEC are plated per well in 6-well plates in 2 ml DMEM as supplemented and under the conditions described in Example 1. When the cells reach 50%-70% confluency, a total of 1.6  $\mu$ g of DNA (comprising test plasmids, reporter constructs and the  $\beta$ -gal reporter) and 8  $\mu$ g of lipofectamine are used to transfect the cells in each well. After incubation of the cells for 5 hours, FCS is added to the cells to make a final concentration of 10%. After incubation for 48 hours, the cells are stimulated by adding to triplicate wells 100 U/ml of TNF $\alpha$  or 100 ng/ml of lipopolysaccharide (LPS) (Sigma E.Coli 0B55). Non-stimulated cells serve as control ("NS" or "control"). Seven hours after stimulation, the cells are harvested (in the following Examples all volume or weight amounts are on a per well basis; the expression "cell population" or "group of cells" refers to the cell population of a single well plate, i.e. estimated to be approximately  $5 \times 10^5$  cells; in the bar graphs, the bars represent the mean of triplicate values; standard error is represented by a bracket).

**Example 2: E-selectin reporter (A20 expression in BAEC inhibits E-selectin induction in a dose-dependent manner)**

BAEC (bovine aortic endothelial cells) are cotransfected with 0.7  $\mu$ g of the porcine E-selectin reporter construct, together with the A20 expression plasmid or the pAC control plasmid or both. The header portion of FIG. 2 indicates the amount of A20 plasmid provided to each cell population, as follows:

lanes 1, 5, 9: 0  $\mu$ g A20;

lanes 2, 6, 10: 0.125  $\mu$ g A20;

lanes 3, 7, 11: 0.5  $\mu$ g A20;

lanes 4, 8, 12: 0.7  $\mu$ g A20.

pAC is titrated with the A20 plasmid where necessary to bring the total concentration of A20 and pAC vector to 0.7  $\mu$ g per well.

FIG. 2 is a bar graph representing the results of a luciferase assay of each group of cells. Induction of the luciferase gene under the control of the E-selectin promoter is correlatable to the amount in relative light units (RLU) detected in the assay. FIG. 2 demonstrates that stimulation of the cells with TNF or LPS results in substantial increases in activity of the E-selectin reporter in the untreated control (lane 1); or in the stimulated

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cells co-transfected with only the pAC control (lanes 5 and 9), where there are 8 and 14-fold increases in E-selectin activity. Stimulated cells transfected with the A20 construct show significant inhibition of induction of the E-selectin reporter (lanes 5 v. 8, 9 v. 12).

It is also apparent that A20 expression inhibits E-selectin induction in a dose-dependent manner: when 0.125  $\mu$ g of A20 are used, the inhibition reaches 53% for TNF-stimulated cells and 78% for LPS-stimulated cells (lane 5 v. 6, 9 v. 10). Virtually complete inhibition is achieved when the amount of A20 used is 0.5  $\mu$ g and higher, as compared to the basal levels detected in the non-stimulated BAEC transfected with the empty vector (lane 1 v. lanes 7, 8, 11 and 12). In addition, A20 expression decreases the basal, unstimulated activity of the E-selectin reporter by 2-fold when used at 0.5  $\mu$ g and higher.

Since maximal inhibition is obtained by transfecting with 0.5 to 0.7  $\mu$ g A20 vector, the concentration of A20 plasmid used to transfect groups of cells in Examples 3, 4 and 5 is selected to be 0.5  $\mu$ g.

**Example 3: IL-8, I $\kappa$ B $\alpha$  (ECI-6) and TF reporter constructs**

BAEC are cotransfected as described in the General Procedure above with 0.5  $\mu$ g of either the A20 expression plasmid or the pAC control plasmid, and 0.7  $\mu$ g of one of the above-indicated reporter constructs, which are up-regulated during EC activation. FIGS. 3A-3C are bar graphs representing the results of a luciferase assay for each reporter transfection (in FIGS. 3A-3C, as well as FIG. 4 and FIG. 5A, the presence ("+") or absence ("-") of A20 or pAC is indicated in the header):

a) **IL-8 reporter**: When the IL-8 reporter is cotransfected with empty pAC vector, luciferase activity increases 2.5 and 2.7-fold after stimulation with TNF $\alpha$  and LPS, respectively (FIG. 3A, lanes 1 v. 3 and 5). However, when the IL-8 reporter is cotransfected with the A20 expression plasmid, luciferase levels after TNF $\alpha$  or LPS stimulation are reduced to below that seen with non-stimulated pAC-transfected cells (60% below the luciferase activity of unstimulated cells, lane 1 v. 4 and 6). Furthermore, A20 overexpression decreases the basal luciferase activity of the IL-8 reporter by 3-fold (FIG. 3A, lane 1 v. 2).

- b) **I $\kappa$ B $\alpha$  reporter**: The results of the co-transfections performed using the porcine I $\kappa$ B $\alpha$  (ECI-6) reporter construct are similar to those seen with IL-8. Induction with TNF $\alpha$  and LPS reaches 1.6 and 3.6-fold, respectively. Inhibition is virtually complete when A20 is cotransfected with the I $\kappa$ B $\alpha$  reporter. TNF $\alpha$ - or LPS- induced luciferase activities are also lower than the basal levels noted with the empty vector (FIG. 3B, lane 1 v. lanes 4 and 6). Co-transfection with A20 is found to decrease by 5-fold the basal level of ECI-6 luciferase activity (FIG. 3B, lanes 1 v. 2).
- c) **Tissue factor reporter**: In a comparable manner, A20 expression inhibits the 3.5 and 4.5-fold induction of TF reporter activity after TNF $\alpha$  and LPS stimulation, respectively (FIG. 3C, lanes 3, 4, 5, 6). However, a decrease in basal TF reporter activity with A20 co-expression is not observed (FIG. 3C, lane 1 v. 2).

#### **Example 4: NF- $\kappa$ B reporter**

BAEC are cotransfected according to the General Procedure with 0.5  $\mu$ g of either the A20 expression plasmid or the pAC control plasmid and 0.7  $\mu$ g of the NF- $\kappa$ B reporter construct, and the results are shown in the bar graph comprising FIG. 4. Results demonstrate that A20 expression abrogates the 12 and 28-fold induction of reporter activity in response to TNF $\alpha$  and LPS, respectively (FIG. 4, lanes 3 v. 4, 5 v. 6). There is no apparent significant difference between the basal levels of luciferase activity between A20 and pAC transfected cells (FIG. 4, lane 1 v. 2).

All the reporters listed above are known to be highly dependent on NF- $\kappa$ B. Activation of these reporters by either LPS or TNF $\alpha$  is found to be inhibited by expression of A20, demonstrating that the inhibitory effect of A20 on EC activation relates, at least in part and perhaps totally, to inhibition of NF- $\kappa$ B.

#### **Example 5: RSV-LUC and HIV-CAT reporters**

To test non-specific or toxic effects of A20 on the transcriptional machinery, cells are transfected according to the General Procedure with a constitutive, non-inducible reporter, RSV-LUC, which is independent of NF- $\kappa$ B. Also tested is the HIV-CAT reporter, which is induced by the viral c-Tat protein through Sp1 rather than NF- $\kappa$ B binding (Zimmermann et al. [1991], *supra*). Cells are transfected with 0.5  $\mu$ g of either A20 or pAC (RSV-LUC reporter) (as shown in the header of FIG. 5A), or A20 titrated

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with pAC to make up a total of 0.5  $\mu$ g (HIV-CAT reporter) (as shown in the header of FIG. 5B). For the RSV-LUC reporter, cell groups are either non-stimulated ("Control") or TNF- or LPS-stimulated. For the HIV-CAT reporter, cells are either unstimulated ("Control") or stimulated with 0.2  $\mu$ g of the c-Tat protein. It is found that basal luciferase activities of the RSV-LUC reporter are comparable to that seen in the A20 and pAC transfected BAEC.

FIGS. 5A-5B are bar graphs representing the results of a luciferase assay. It is apparent that no significant induction is achieved upon TNF or LPS stimulation in either the pAC- or the A20-expressing cells; luciferase values remain comparable among the 2 groups (FIG. 5A). With regard to HIV-CAT, the results demonstrate that A20 expression affects neither the basal levels nor the 10 to 15-fold induction of the reporter observed upon stimulation with c-Tat (FIG. 5B, lane 1 v. lanes 2, 3, 4 and lane 1 v. lanes 6, 7, 8).

The above demonstrates that expression of A20 prevents gene induction associated with endothelial cell activation. Reporter inhibition is seen when either TNF or LPS is used to stimulate the EC, pointing to the broad inhibitory effect of A20 on gene induction. The similar effect on LPS- and TNF-induced signaling also excludes any specific association of the action of A20 with the TNF response per se. The basal expression of the E-selectin, IL-8 and I $\kappa$ B $\alpha$  reporters is also significantly decreased in cells expressing A20. Inhibition is found to be dose-dependent.

Expression of A20 has no apparent effect on either the constitutive activity of the RSV-LUC reporter or the c-Tat stimulation of the HIV-CAT reporter, which also demonstrates a lack of effect of A20 on Sp1, which illustrates the specificity of A20 in blocking NF- $\kappa$ B activation.

Therefore in addition to its ability to protect cells from apoptosis, expression of A20 inhibits NF- $\kappa$ B activation, and thereby inhibits gene induction. This function places A20 in the category of genes that are dependent on NF- $\kappa$ B for their induction, but that subsequently inhibit NF- $\kappa$ B and thus, endothelial cell activation. Such genes presumably function in negative regulatory loops to regulate the extent and duration of endothelial cell activation.

While not intending to be bound thereby, it is proposed that an alternative mechanism exists by which A20 functions as an antioxidant. The full-length human A20 cDNA encodes 7 Cys2/Cys2 repeats, which characterizes it as a Zn finger protein with a

potentially high Zn binding capacity (Opipari et al. [1990], *supra*). Zn can act as an antioxidant by two mechanisms: the protection of sulfhydryl groups against oxidation and the inhibition of the production of reactive oxygens by transition metals, mainly iron and copper. There is evidence that antioxidants such as PDTC can prevent gene induction associated with EC activation, by inhibition of NF- $\kappa$ B (E.B. Cunningham, *Biochem.Biophys.Res.Comm.* 215 [1995] 212-218) and also to prevent TNF-mediated apoptosis (T.M. Buttke and P.A. Sandstrom, *Immunol. Today* 15 [1994] 7-10). These findings correlate with the fact that signaling via the TNF receptor results in a rapid rise in the levels of intracellular reactive oxygen intermediates that cause apoptosis via oxidative damage (Buttke and Sandstrom [1994], *supra*).

**Example 6: Adenoviral-mediated transfer of A20 to porcine aortic endothelial cells**

A recombinant A20 adenovirus (rAd.A20) is constructed by homologous recombination between a transfer vector containing the human A20 cDNA, pAC.CMV.NLS-A20, and pJM17, a plasmid-borne form of the adenovirus 5 genome. The encoded A20 protein is unmodified. Homologous recombination is performed in 293 cells. Clonal viruses are obtained by limiting dilution cloning in 96-well plates, and analyzed by Northern blotting for the presence of A20 mRNA. After identification of a positive recombinant A20 adenovirus, amplification is performed in 293 cells. Cesium chloride purified adenovirus is used to infect porcine aortic endothelial cells (PAEC) at a multiplicity of infection (MOI) of 500 to 2500/cell. A20 infection is checked by Northern blot analysis of infected cells. 48 hours after infection, cells are stimulated with 100 U/ml of TNF or 100 ng/ml of LPS. mRNA is extracted 2-6 hours following EC stimulation. Northern blot analysis shows that A20 adenovirus-infected cells abrogate by 60-90% the TNF- and LPS-mediated induction of E-selectin, IL-8, and I $\kappa$ B $\alpha$ . The percentage of inhibition is directly correlated to mRNA levels of A20 detected in infected cells. In accordance with Northern blot analysis, A20 expression in PAEC inhibits by up to 90% the surface expression of E-selectin as assessed by ELISA. Mock-infected cells as well as PAEC infected with a  $\beta$ -galactosidase rAD are used as controls. These results further demonstrate that expression of A20 inhibits EC activation.

**Example 7: Co-transfer of BAEC with Bcl-2 and Bcl-x<sub>L</sub> expression plasmids along with reporter constructs**

Approximately  $3 \times 10^5$  bovine aortic endothelial cells obtained from culture in 10 cm plates as described in Example 1, are plated per well in a 6-well plate in 2 ml of DMEM as supplemented and under the conditions described in Example 1. When the cells reach 50%-70% confluency, a total of 1.5-1.6  $\mu\text{g}/\text{well}$  of DNA (test plasmids and reporter constructs) is added to 8 mg of lipofectamine per well and incubated at room temperature for 30 minutes before being added to the cells in triplicate. In all experiments, BAEC are co-transfected with 0.5  $\mu\text{g}$  of Bcl-2, Bcl-x<sub>L</sub> or pAC, and 0.7  $\mu\text{g}$  of the E-selectin, ECI-6 (IkB $\alpha$ ) or NF- $\kappa$ B - luciferase (luc) reporters, as well as 0.3  $\mu\text{g}$  of the  $\beta$ -galactosidase (b-gal) reporter. After 5 hours incubation, FCS is added to the medium to achieve a final concentration of 10%. 48 hours thereafter the cells are stimulated with either human recombinant TNF (100U/ml) or LPS (100ng/ml), and are harvested 7 h after stimulation.

The effect of BCL-2 and BCL-X<sub>L</sub> expression upon EC activation is first studied using an endothelial cell-specific marker, E-selectin. BAEC ( $3 \times 10^5$  to  $5 \times 10^5$  cells) are co-transfected with the porcine E-selectin reporter construct (0.7  $\mu\text{g}$ ) as well as the bcl-2, the bcl-x<sub>L</sub> expression plasmids (0.5  $\mu\text{g}$ ) or the pAC control (0.5  $\mu\text{g}$ ) plasmid in conjunction with the RSV  $\beta$ -gal plasmid (0.3  $\mu\text{g}$ ).

The results, depicted in FIG. 6A, show that BCL-2 and BCL-X<sub>L</sub> overexpression leads to a significant decrease in the luciferase activity of the E-selectin reporter after both TNF and LPS stimulation. In the pAC control, induction with either TNF or LPS leads to a 35- and 50-fold increase in the activity of the E-selectin reporter, respectively. BCL-X<sub>L</sub> expression inhibits TNF- and LPS-induced luciferase activity very significantly, this inhibition reaching respectively 95% and 90% of the control following TNF and LPS stimulation (lanes 4 and 7 v. 5, 8). Inhibition is seen to be complete when BCL-2 is expressed in the cells. No induction of the E-selectin reporter is seen upon TNF and LPS stimulation (lanes 4 and 7 v. lanes 6 and 9). The basal level of luciferase activity of the E-selectin reporter is not affected by BCL-2 or BCL-X<sub>L</sub> expression.

The results of the co-transfections performed using the porcine IkB $\alpha$  (ECI-6) reporter construct (FIG. 6B) are similar to those seen with E-selectin. Induction with TNF and LPS reaches 2.5 fold (lanes 1 v. 4 and 6). BCL-X<sub>L</sub> and BCL-2 expression



completely abolishes TNF- and LPS-induced luciferase activity following TNF and LPS stimulation (lanes 4 and 7 v. 5, 6 and 7, 8). The basal level of luciferase activity of the  $\text{I}\kappa\text{B}\alpha$  reporter is not affected by BCL-2 or BCL-X<sub>L</sub> expression.

BAEC are co-transfected with an NF- $\kappa\text{B}$  reporter construct that is solely dependent upon NF- $\kappa\text{B}$ , and either bcl-x<sub>L</sub>, bcl-2 or the empty vector, pAC (FIG. 6C). BCL-X<sub>L</sub> expression significantly decreases the 10- and 26-fold induction of reporter activity in response to TNF and LPS, respectively (lanes 4 and 7 v. 5 and 8). This inhibition reaches 50% and 70%, respectively. In contrast with BCL-X<sub>L</sub>, BCL-2 expression totally abrogates the TNF and LPS inducibility of the NF- $\kappa\text{B}$  reporter (lanes 4 and 7 v. 6 and 9). There appears to be no significant difference in the basal levels of luciferase activity between BCL-X<sub>L</sub>, BCL-2 and pAC (lanes 1 v. 2 and 3).

Therefore the demonstrated EC inhibition is shown to be related to inhibition of the transcriptional factor NF- $\kappa\text{B}$ .

#### **Example 8: A20 mutants**

A truncation of the A20 gene from bp 1182 to 2450 and spanning the 7 Zn binding domains of the molecule is obtained by digestion of the 2.4 kB cDNA with NcoI. This fragment is expressed as a polypeptide of 417 amino acid residues (residues 373 to 790 of SEQ. ID. NO. 1). The truncated A20 gene is cloned into pBac 4 (Promega) and then subcloned into the pAC expression vector to be used in co-transfection experiments in BAEC. In these experiments,  $2 \times 10^5$  BAEC are plated per well in a 6-well plate with 2 ml of medium as described above. Cells are transfected once they reach 50-70% confluence. 1.5-1.6  $\mu\text{g}$ /well of DNA (test plasmids and reporter constructs) are added to 4 units of lipofectamine per well and incubated at room temperature for 30 minutes before being added to the cells in triplicate. In this experiment, 0.3  $\mu\text{g}$  of the  $\beta$ -gal reporter is used, with 0.5  $\mu\text{g}$  of: A20, or truncated A20 (tA20), or the control plasmid pAC, and 0.7  $\mu\text{g}$  of the E-selectin-luc reporter. 48 hours after transfection, cells are challenged with either 100 U/ml of TNF or 100 ng/ml of LPS. Cell extracts are prepared 7 hours after stimulation and assayed for  $\beta$ -galactosidase and luciferase expression, as above. Two clones expressing the truncated form of the A20 are tested: clone #3 and #7.

FIG. 7 shows that expression of the truncated form of A20, i.e. consisting essentially of the 7 Zn binding domains of the molecule, inhibits as efficiently as A20, the induction of the E-selectin reporter upon stimulation by TNF or LPS.

**Example 9: Reguable gene expression in transgenic mice**

**a) Inducible tetracycline expression system:**

A system for temporal regulation of anti-apoptotic gene expression is highly desirable to inhibit NF- $\kappa$ B activation on a controllable basis.

An inducible expression system can be employed to regulate anti-apoptotic gene expression in vivo, in particular the binary plasmid system described by Gossen and Bujard, *PNAS* [1992], *supra*, which is inducible by the withdrawal of tetracycline; or the tetracycline-dependent system disclosed by Furth et al., *PNAS* [1994], *supra*. For example the Gossen and Bujard system employs a first plasmid containing a bacterial, tetracycline-sensitive DNA binding protein fused to the HSV-VP16 transcriptional activation domain (tTA) expressed from a constitutive CMV promoter. A second plasmid contains 7 copies of the binding site for tTA, downstream of which the anti-apoptotic gene is cloned into the vector. When both plasmids are present in a cell, the tTA protein drives high level transcription of the anti-apoptotic gene of the invention. In the presence of tetracycline there is no expression of the anti-apoptotic transgene. In the absence of tetracycline, there is high level expression of the anti-apoptotic gene (in the Furth et al. system, the presence of tetracycline promotes expression of the anti-apoptotic gene, whereas in the absence of tetracycline there is no expression of the anti-apoptotic transgene).

**b) Transgenic mice:**

For the generation of transgenic mice the anti-apoptotic gene is cloned into a suitable vector, for example, as described by Gossen and Bujard, *PNAS* [1992] *supra*. Two separate founder strains are generated for tTA and the anti-apoptotic gene. Transgenic mice of each strain are rendered homozygous by crossing heterozygous animals. Homozygous animals of each strain are bred as lines. Crossing tTA/tTA mice with, e.g., bcl-2/bcl-2 mice results in double transgenic mice carrying both tTA and Bcl-2 transgenes. These crossings are carried out under cover of tetracycline to prevent anti-apoptotic transgene expression during embryogenesis. Mice carrying the tTA and anti-

apoptotic transgene, respectively, are identified by Southern blotting to prevent expression of the anti-apoptotic gene during embryogenesis.

Mice that express the anti-apoptotic gene in EC can be used as donors for xenotransplantation (heart and/or kidney) into rats for modelling purposes.

**Example 10: Generation of transgenic pigs**

A transgenic pig expressing a human anti-apoptotic gene (e.g., A20, bcl-2, bcl-x<sub>L</sub>, A1) is prepared by techniques disclosed in Pinckert et al. [1994], *supra*.

**Example 11: Adenoviral-mediated BCL-2 expression inhibits NF- $\kappa$ B activation**

Nuclear extracts are prepared from rAd.Bcl-2 or rAd. $\beta$ -gal-infected PAEC before, and two hours following, treatment with TNF (100U/ml). NF- $\kappa$ B activation and binding to a  $\kappa$ B binding oligonucleotide derived from the human Immunoglobulin (Ig)  $\kappa$  promoter is evaluated by electrophoretic mobility shift assay (EMSA) (FIG. 8).

Nuclear extracts from PAEC expressing BCL-2 reveal little constitutive, and no inducible, binding of NF- $\kappa$ B, whereas rAd. $\beta$ -gal - infected cells demonstrate strong induction of NF- $\kappa$ B binding activity following TNF stimulation. Specificity of DNA binding is confirmed by the use of excess cold wild-type (specific competitor) or a non-specific competitor (AP-1) probe as controls (lanes 3 and 4).

**Example 12: BCL-2 expression in PAEC inhibits I $\kappa$ B $\alpha$  degradation following TNF treatment**

Cytoplasmic extracts are prepared prior to, as well as ten minutes or two hours following, TNF treatment of rAd.Bcl-2 - or rAd. $\beta$ -gal - infected PAEC. Protein concentration of the cytoplasmic extracts is quantitated by the Bradford method. I $\kappa$ B $\alpha$  expression is evaluated by Western blot. I $\kappa$ B $\alpha$  is detected using anti-MAD-3 rabbit polyclonal IgG anti-serum (Santa-Cruz Biotechnology, Santa Cruz, CA, USA) and peroxidase-conjugated goat anti-rabbit secondary antibody followed by enhanced chemiluminescence (ECL) detection (Amersham Corp.).

Results show that BCL-2 expression in PAEC inhibits the usual I $\kappa$ B $\alpha$  degradation that occurs 10 minutes following TNF stimulation. Results shown are representative of 3 independent experiments (FIG. 9).

**Example 13: BCL-2 expression in the EC does not affect binding of the transcription factor, cAMP responsive element (CRE)**

To determine whether BCL-2 expression affects nuclear binding to a CRE probe, nuclear extracts are prepared from rAd.Bcl-2- or rAd. $\beta$ -gal - infected PAEC before, and two hours following, treatment with TNF (100U/ml) and assayed by EMSA (electrophoretic mobility shift assay) for their binding activity of a radio-labeled CRE oligonucleotide. No difference is observed between the Bcl-2- and the  $\beta$ -gal - infected cells (FIG. 10).

**Example 14: Function of the Bcl gene A1 in endothelial cells**

**a) A1 expression in EC inhibits TNF- and LPS-induced activation through inhibition of NF- $\kappa$ B:**

HUVEC, when stimulated with TNF, express A1. The maximum induction at the mRNA level occurs at approximately three hours following TNF stimulation. Expression of A1 in the EC inhibits activation following TNF and LPS treatment; this inhibitory effect relates to inhibition of NF- $\kappa$ B activation. BAEC are co-transfected with an expression plasmid encoding for A1 and reporter constructs comprising the promoter region of E-selectin linked to the luciferase gene and a reporter solely dependent upon NF- $\kappa$ B for its induction (FIG. 11).

**b) Expression of A1 is dependent on NF- $\kappa$ B:**

To evaluate whether functional NF- $\kappa$ B activity is needed for the induction of A1, it is investigated whether A1 continues to be inducible following TNF stimulation in HUVEC even in the presence of an overexpressed inhibitor of NF- $\kappa$ B (i.e. I $\kappa$ B $\alpha$  or A20). HUVEC are infected with the rAd.I $\kappa$ B $\alpha$ , rAd.A20 or the control rAd. $\beta$ -gal at an MOI of 100. Northern blot reveals high levels of I $\kappa$ B $\alpha$  and of A20 mRNA in the cells. Forty-eight hours following infection, EC are stimulated with 100U of TNF for three hours. RNA is extracted. Expression of A1 is analyzed by Northern blot analysis.

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Results demonstrate that expression of  $\text{I}\kappa\text{B}\alpha$  or of A20 inhibits the induction of A1 messenger RNA as seen in the control rAd. $\beta$ -gal-infected cells. Similarly, induction of  $\text{I}\kappa\text{B}\alpha$  (another NF-B dependent gene) is inhibited in the A20-expressing cells as compared to controls, further confirming the ability of A20 to block up-regulation of NF- $\kappa\text{B}$  dependent genes (FIG. 12).

## SEQUENCE LISTING

### (I) GENERAL INFORMATION:

#### (i) APPLICANT:

- (A) NAME: Novartis AG
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- (G) TELEPHONE: 61-324 5269
- (H) TELEFAX: 61-322 7366

- (ii) TITLE OF INVENTION: ANTI-APOPTOTIC GENE THERAPY FOR  
TRANSPLANTATION AND INFLAMMATORY  
CONDITIONS

- (iii) NUMBER OF SEQUENCES: 5

#### (iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: PatentIn Release #1.0, Version #1.25 (EPO)

#### (v) CURRENT APPLICATION DATA:

APPLICATION NUMBER: WO PCT/EP97/....

#### (vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/601515
- (B) FILING DATE: 14-FEB-1996

#### (vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/634995
- (B) FILING DATE: 19-APR-1996

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(2) INFORMATION FOR SEQ ID NO. 1:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 790 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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Thr Asn Gly Ile Ile His His Phe Lys Thr Met His Arg Tyr Thr Leu
35          40          45
Glu Met Phe Arg Thr Cys Gln Phe Cys Pro Gln Phe Arg Glu Ile Ile
50          55          60
His Lys Ala Leu Ile Asp Arg Asn Ile Gln Ala Thr Leu Glu Ser Gln
65          70          75          80
Lys Lys Leu Asn Trp Cys Arg Glu Val Arg Lys Leu Val Ala Leu Lys
85          90          95
Thr Asn Gly Asp Gly Asn Cys Leu Met His Ala Thr Ser Gln Tyr Met
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165         170         175
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Glu Ile His Ile Phe Val Leu Cys Asn Ile Leu Arg Arg Pro Ile Ile
195         200         205

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 Tyr Lys Lys Trp Gln Glu Asn Ser Glu Gln Gly Arg Arg Glu Gly His  
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 Asn Pro Glu Glu Ser Thr Gly Gly Pro His Ser Ala Pro Pro Thr Ala  
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 465 470 475 480  
 Pro Gly Cys Pro Phe Thr Leu Asn Val Gln His Asn Gly Phe Cys Glu  
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 Thr Arg His Leu Asp Pro Gly Lys Cys Gln Ala Cys Leu Gln Asp Val  
 515 520 525





## (2) INFORMATION FOR SEQ ID NO. 2:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4440 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: double

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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CAGTTTCGGG AGATCATCCA CAAAGCCCTC ATCGACAGAA ACATCCAGGC CACCCCTGGAA	300
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GGTGACGGCA ATTGCCCTCAT GCATGCCACT TCTCAGTACA TGTGGGCGT TCAGGACACA	420
GACTTGGTAC TGAGGAAGGC GCTGTTTCAGC ACGTCAAGG AAACAGACAC ACGCAACTTT	480
AAATCCCGCT GGCAACTGGA GTCTCTCAAA TCTCAGGAAT TTGTTGAAAC GGGGCTTTGC	540

TATGATACTC GGAACCTGGAA TGATGAATGG GACAACTCTTA TCAAAATGGC TTCCACAGAC 600  
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CTTTGCAACA TCCTCAGAAG GCCAATCAAT GTCAATTCAG ACAAATGCT AAGAAGTTTG 720  
GAATCAGGTT CCAATTTGCG CCTTTGAAA GTGGGTGGAA TTTACTTGCC TCTCCACTGG 780  
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CCCTTGGTGA CCCTGAAGGA CAGTGGGCCT GAAATCCGAG CTGTTCCACT TGTAAACAGA 900  
GACCGGGGAA GATTTGAAGA CTTAAAAGTT CACTTTTGA CAGATCCTGA AAATGAGATG 960  
AAGGAGAAGC TCTTAAAAGA GTACTTAATG GTGATAGAAA TCCCCGTCCA AGGCTGGGAC 1020  
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AGGAGCCCCG GCTGCCCCCTT CACACTGAAT GTGCAGCACA ACGGATTTTG TGAACGTTGC 1560  
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TGCCACAGAG CTGGAAACGA CGCCCTGCT GGCTGCCCTGT CTCAAGCTGC ACGGACTCCT 1860  
GGGACAGGA CGGGACGAG CAAGTGCAGA AAAGCCGGCT GCGTGTATTT TGGGACTCCA 1920  
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3180 TAAAAAATTA GAATATTTTA CTGGGAAGAC GTGTAACCTCT TTGGGTTATT ACTGTCTTTA  
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3840 CACAGGGAAA GATGTGGCCT TTTGTGATGG TTTTATTTTC TGTAAACACT GTGTCCCTGG  
3900 GGGGCTGGGA AGTCCCCCTGC ATCCCATGGT ACCCTGGTAT TGGGACAGCA AAAGCCAGTA  
3960 ACCATGAGTA TGAGGAAATC TCCTTCTGT TCTGGCTTAC AGTTTCTCTG TGTGCTTTGT

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AGAGATAAAG GCTGCCATTT TGGGGGTCTG TACTTATGGC CTGAAAAATAT TTGTGATCCA 4080  
TAACTCTACA CAGCCTTTAC TCATACTATT AGGCACACTT TCCCCTTAGA GCCCCCTAAG 4140  
TTTTTCCCAG ACGAATCTTT ATAATTTCCT TTCCAAAGAT ACCAAATAAA CTTCAGTGTT 4200  
TTCATCTAAT TCTCTTAAAG TTGATATCTT AATATTTTGT GTTGATCATT ATTTCCATTC 4260  
TTAATGTGAA AAAAAAGTAAT TATTATACT TATTATAAAA AGTATTGAA ATTTGCACAT 4320  
TTAATGTGCC CTAATAGAAA GCCACCTATT CTTTGTGGA TTTCTTCAAG TTTTTCCTAAA 4380  
TAAATGTAAC TTTTTCACAAG AGTCAACATT AAAAAATAAA TTATTTAAAA AAAAAAATAA 4440

-45-

**(2) INFORMATION FOR SEQ ID NO. 3:****(i) SEQUENCE CHARACTERISTICS:**

(A) LENGTH: 205 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

**(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:**

Met	Ala	His	Ala	Gly	Arg	Thr	Gly	Tyr	Asp	Asn	Arg	Glu	Ile	Val	Met	1	5	10	15
Lys	Tyr	Ile	His	Tyr	Lys	Leu	Ser	Gln	Arg	Gly	Tyr	Glu	Trp	Asp	Ala	20	25	30	
Gly	Asp	Val	Gly	Ala	Ala	Pro	Pro	Gly	Ala	Ala	Pro	Ala	Pro	Gly	Ile	35	40	45	
Phe	Ser	Ser	Gln	Pro	Gly	His	Thr	Pro	His	Pro	Ala	Ala	Ser	Arg	Asp	50	55	60	
Pro	Val	Ala	Arg	Thr	Ser	Pro	Leu	Gln	Thr	Pro	Ala	Ala	Pro	Gly	Ala	65	70	75	80
Ala	Ala	Gly	Pro	Ala	Leu	Ser	Pro	Val	Pro	Pro	Val	Val	His	Leu	Ala	85	90	95	
Leu	Arg	Gln	Ala	Gly	Asp	Asp	Phe	Ser	Arg	Arg	Tyr	Arg	Gly	Asp	Phe	100	105	110	
Ala	Glu	Met	Ser	Ser	Gln	Leu	His	Leu	Thr	Pro	Phe	Thr	Ala	Arg	Gly	115	120	125	
Arg	Phe	Ala	Thr	Val	Val	Glu	Glu	Leu	Phe	Arg	Asp	Gly	Val	Asn	Trp	130	135	140	
Gly	Arg	Ile	Val	Ala	Phe	Phe	Glu	Phe	Gly	Gly	Val	Met	Cys	Val	Glu	145	150	155	160
Ser	Val	Asn	Arg	Glu	Met	Ser	Pro	Leu	Val	Asp	Asn	Ile	Ala	Leu	Trp	165	170	175	
Met	Thr	Glu	Tyr	Leu	Asn	Arg	His	Leu	His	Thr	Trp	Ile	Gln	Asp	Asn	180	185	190	
Gly	Gly	Trp	Val	Gly	Ala	Ser	Gly	Asp	Val	Ser	Leu	Gly				195	200	205	

**(2) INFORMATION FOR SEQ ID NO. 4:****(i) SEQUENCE CHARACTERISTICS:**

(A) LENGTH: 233 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

**(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:**

Met	Ser	Gln	Ser	Asn	Arg	Glu	Leu	Val	Val	Asp	Phe	Leu	Ser	Tyr	Lys	1	5	10	15
Leu	Ser	Gln	Lys	Gly	Tyr	Ser	Trp	Ser	Gln	Phe	Ser	Asp	Val	Glu	Glu	20	25	30	35
Asn	Arg	Thr	Glu	Ala	Pro	Glu	Gly	Thr	Glu	Ser	Glu	Met	Glu	Thr	Pro	40	45	50	55
Ser	Ala	Ile	Asn	Gly	Asn	Pro	Ser	Trp	His	Leu	Ala	Asp	Ser	Pro	Ala	60	65	70	75
Val	Asn	Gly	Ala	Thr	Gly	His	Ser	Ser	Ser	Leu	Asp	Ala	Arg	Glu	Val	80	85	90	95
Ile	Pro	Met	Ala	Ala	Val	Lys	Gln	Ala	Leu	Arg	Glu	Ala	Gly	Asp	Glu	100	105	110	115
Phe	Glu	Leu	Arg	Tyr	Arg	Arg	Ala	Phe	Ser	Asp	Leu	Thr	Ser	Gln	Leu	120	125	130	135
His	Ile	Thr	Pro	Gly	Thr	Ala	Tyr	Gln	Ser	Phe	Glu	Gln	Val	Val	Asn	140	145	150	155
Glu	Leu	Phe	Arg	Asp	Gly	Val	Asn	Trp	Gly	Arg	Ile	Val	Ala	Phe	Phe	160	165	170	175
Ser	Phe	Gly	Gly	Ala	Leu	Cys	Val	Glu	Ser	Val	Asp	Lys	Glu	Met	Gln	180	185	190	195
Val	Leu	Val	Ser	Arg	Ile	Ala	Ala	Trp	Met	Ala	Thr	Tyr	Leu	Asn	Asp	200	205	210	215
His	Leu	Glu	Pro	Trp	Ile	Gln	Glu	Asn	Gly	Gly	Trp	Asp	Thr	Phe	Val	220	225	230	
Glu	Leu	Tyr	Gly	Asn	Asn	Ala	Ala	Ala	Glu	Ser	Arg	Lys	Gly	Gln	Glu				
Arg	Phe	Asn	Arg	Trp	Phe	Leu	Thr	Gly	Met	Thr	Val	Ala	Gly	Val	Val				
Leu	Leu	Gly	Ser	Leu	Phe	Ser	Arg	Lys											



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(2) INFORMATION FOR SEQ ID NO. 5:

## (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 175 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iii) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Met	Thr	Asp	Cys	Glu	Phe	Gly	Tyr	Ile	Tyr	Arg	Leu	Ala	Gln	Asp	Tyr	1	5	10	15
Leu	Gln	Cys	Val	Leu	Gln	Ile	Pro	Gln	Pro	Gly	Ser	Gly	Pro	Ser	Lys	20	25	30	
Thr	Ser	Arg	Val	Leu	Gln	Asn	Val	Ala	Phe	Ser	Val	Gln	Lys	Glu	Val	35	40	45	
Glu	Lys	Asn	Leu	Lys	Ser	Cys	Leu	Asp	Asn	Val	Asn	Val	Val	Ser	Val	50	55	60	
Asp	Thr	Ala	Arg	Thr	Leu	Phe	Asn	Gln	Val	Met	Glu	Lys	Glu	Phe	Glu	65	70	75	80
Asp	Gly	Ile	Ile	Asn	Trp	Gly	Arg	Ile	Val	Thr	Ile	Phe	Ala	Phe	Glu	85	90	95	
Gly	Ile	Leu	Ile	Lys	Lys	Leu	Leu	Arg	Gln	Gln	Ile	Ala	Pro	Asp	Val	100	105	110	
Asp	Thr	Tyr	Lys	Glu	Ile	Ser	Tyr	Phe	Val	Ala	Glu	Phe	Ile	Met	Asn	115	120	125	
Asn	Thr	Gly	Glu	Trp	Ile	Arg	Gln	Asn	Gly	Gly	Trp	Glu	Asn	Gly	Phe	130	135	140	
Val	Lys	Lys	Phe	Glu	Pro	Lys	Ser	Gly	Trp	Met	Thr	Phe	Leu	Glu	Val	145	150	155	160
Thr	Gly	Lys	Ile	Cys	Glu	Met	Leu	Ser	Leu	Leu	Lys	Gln	Tyr	Cys	165	170	175		

**Claims:**

1. A mammalian endothelial cell which is genetically modified to express an anti-apoptotic protein which is capable of inhibiting NF- $\kappa$ B activation in the presence of a cellular activating stimulus.
2. A donor endothelial cell, or a tissue or organ comprising such a cell, wherein the cell is genetically modified to regulably or constitutively express an anti-apoptotic protein in a graft recipient, whereby NF- $\kappa$ B is substantially inhibited, for transplantation into a recipient species.
3. A method of genetically modifying a mammalian endothelial cell to render it less susceptible to an inflammatory or other immunological activation stimulus, which comprises inserting in that cell, or a progenitor thereof, DNA encoding an anti-apoptotic protein capable of inhibiting NF- $\kappa$ B and expressing the protein, whereby NF- $\kappa$ B activation in the cell is substantially inhibited in the presence of a cellular activating stimulus.
4. A method of inhibiting cellular activation in a mammalian subject susceptible to an inflammatory or immunological stimulus which comprises genetically modifying endothelial cells of the subject, by insertion of DNA encoding an anti-apoptotic protein capable of inhibiting NF- $\kappa$ B and expressing that protein, whereby NF- $\kappa$ B is substantially inhibited in the cells in the presence of a cellular activating stimulus.
5. A method of transplanting donor endothelial or other mammalian cells, or graftable tissues or organs comprising such cells, to a mammalian recipient in whose blood or plasma these cells, tissues or organs are subject to activation, which comprises:
  - (a) genetically modifying the donor cells, or progenitor cells thereof, by inserting therein DNA encoding an anti-apoptotic protein capable of inhibiting NF- $\kappa$ B; and

(b) transplanting the resultant modified donor cells, or tissues or organ comprising these cells, into the recipient, and expressing in the cells the anti-apoptotic protein, whereby NF- $\kappa$ B activation in the cells is substantially inhibited in the presence of a cellular activating stimulus.

6. A cell according to claim 1 or 2 or a method according to any one of claims 3 to 5 wherein the anti-apoptotic protein is

- a polypeptide having activity of an A20 protein; or
- a polypeptide having activity of BCL-2 protein, a homodimer of that polypeptide, or a heterodimer of that polypeptide and another anti-apoptotic polypeptide of the BCL family; or
- a polypeptide having activity of BCL-X<sub>L</sub> protein, a homodimer of that polypeptide, or a heterodimer of that polypeptide and another anti-apoptotic polypeptide of the BCL family; or
- a polypeptide having activity of A1 protein, a homodimer of that polypeptide, or a heterodimer of that polypeptide and another anti-apoptotic polypeptide of the BCL family.

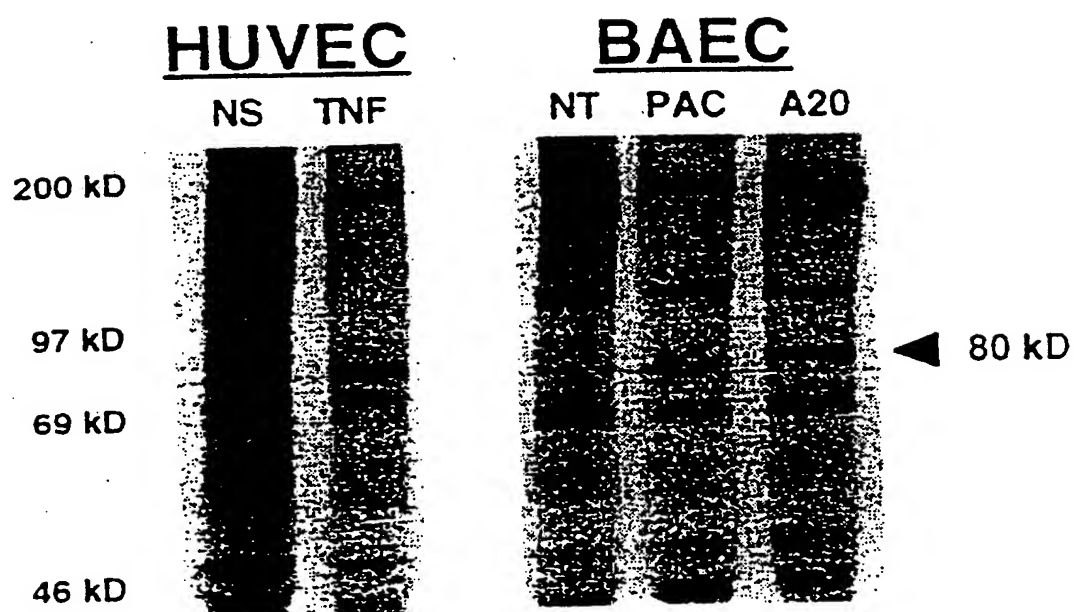
7. A cell according to claim 1 or 2 which is porcine.

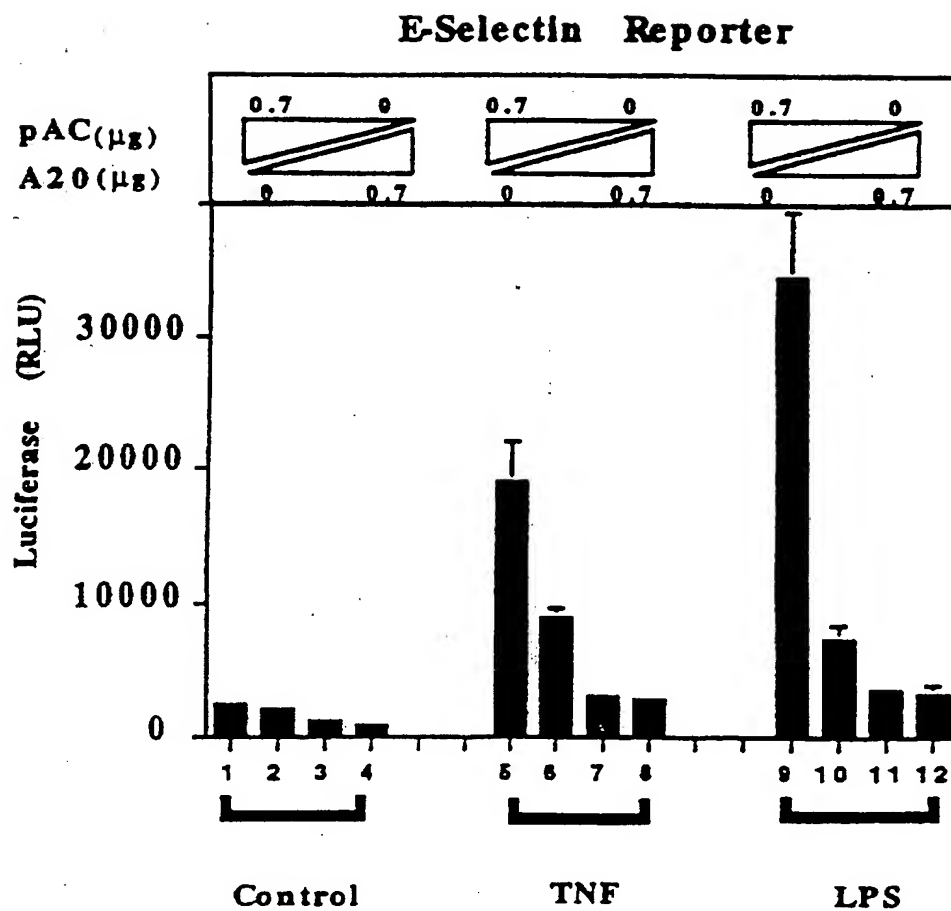
8. A cell according to claim 1 or 2 which is human.

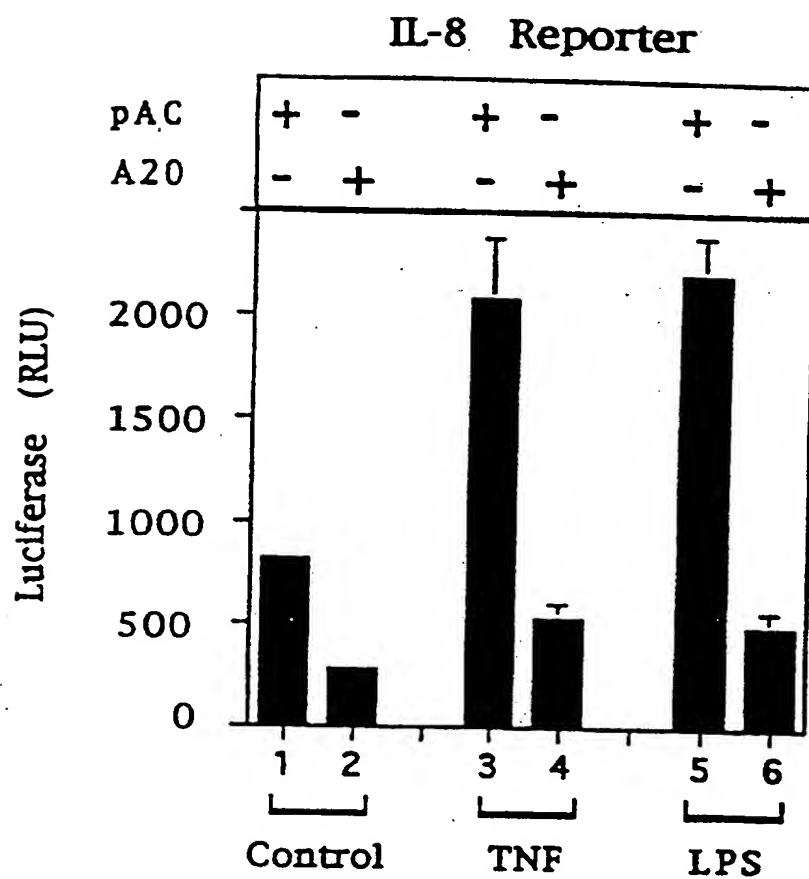
9. A non-human transgenic or somatic recombinant mammal comprising DNA encoding an anti-apoptotic protein of a different species.

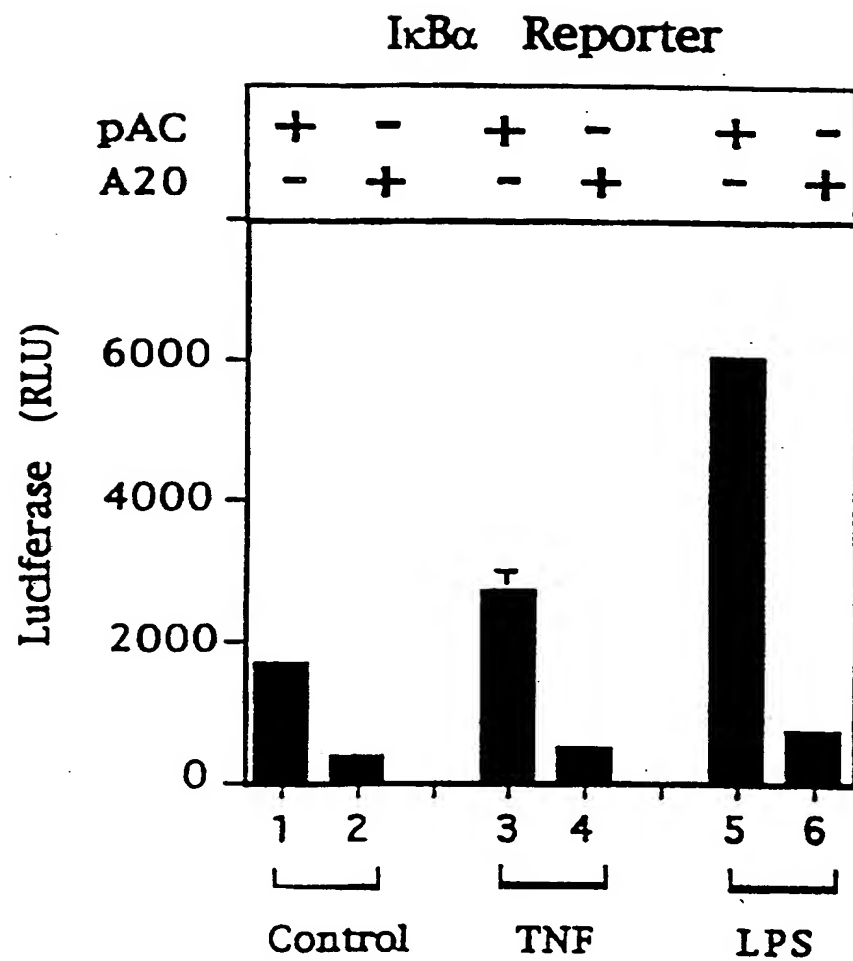
10. A mammal according to claim 9 which is porcine.

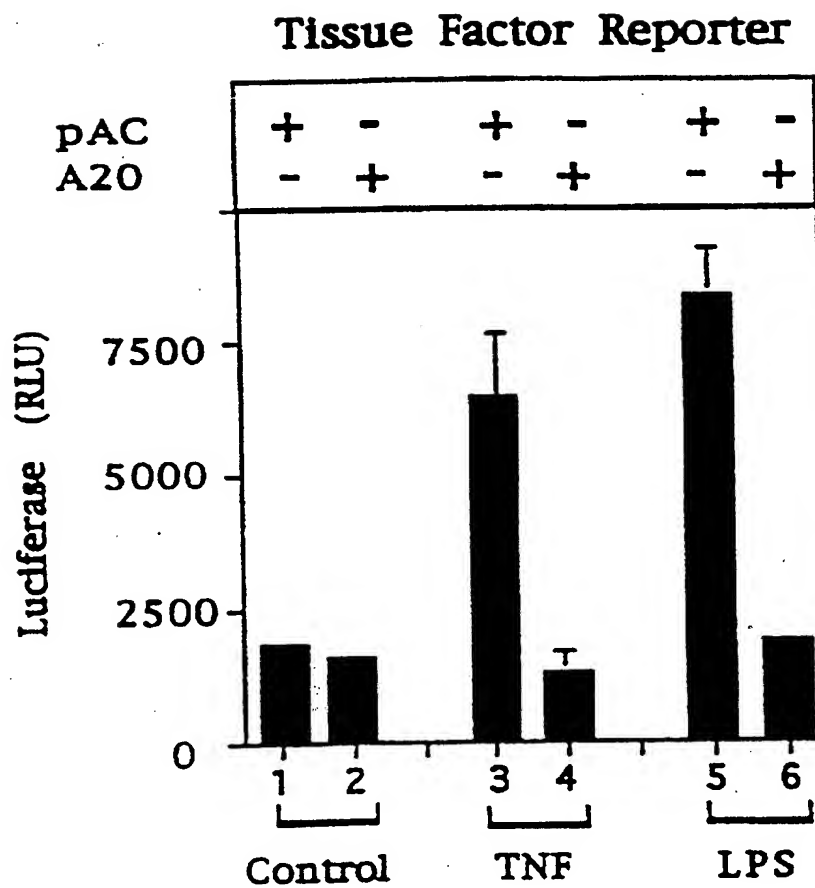
11. A mammal according to claim 10 wherein the anti-apoptotic protein is human.



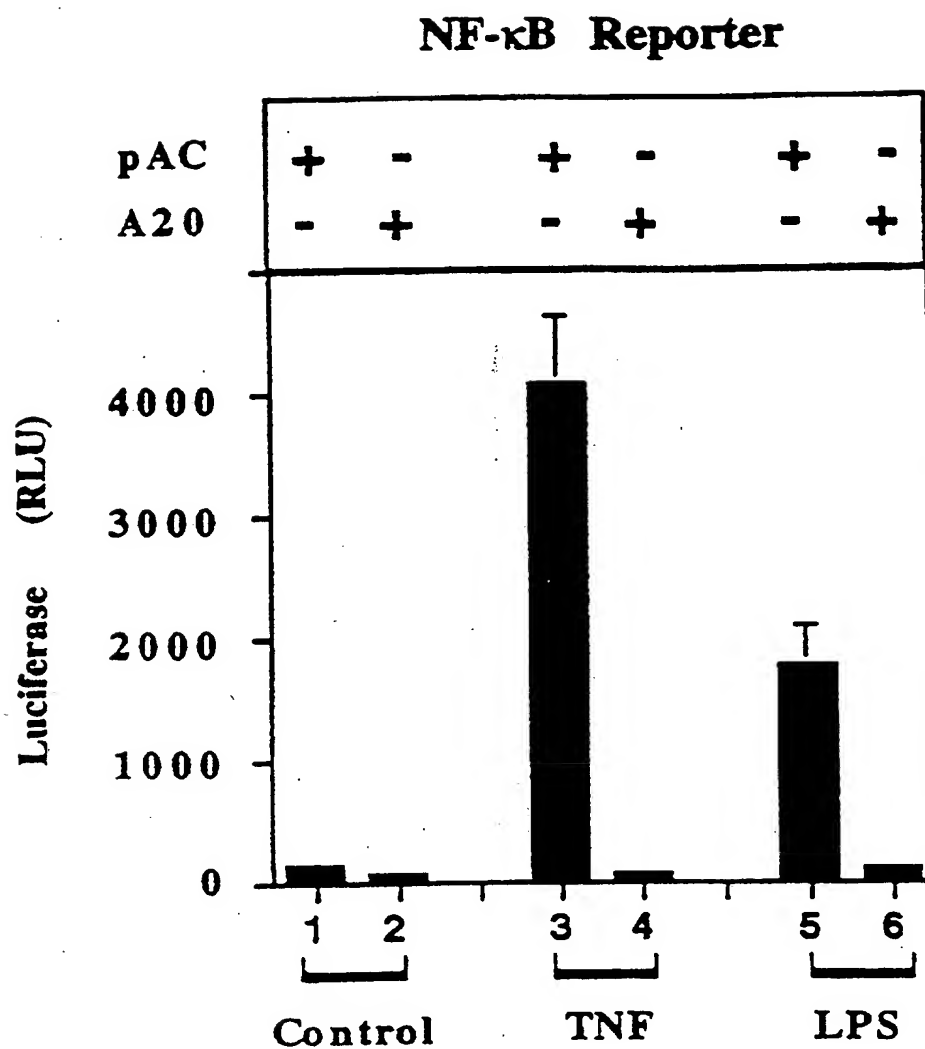


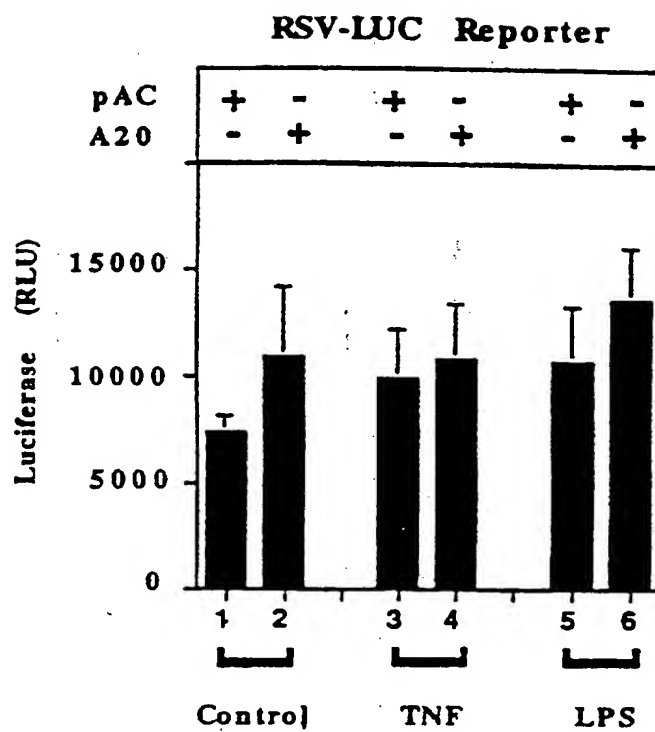












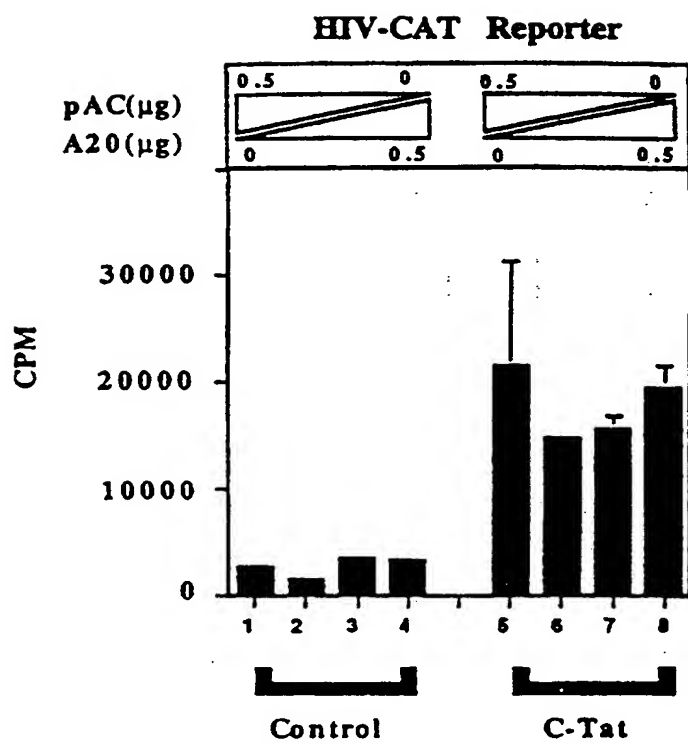
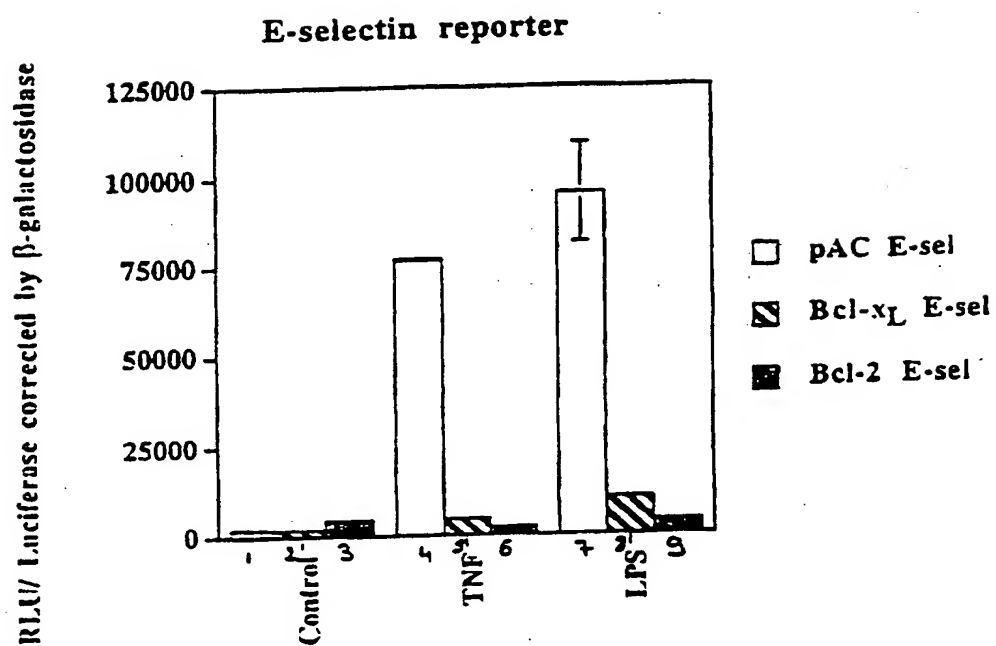
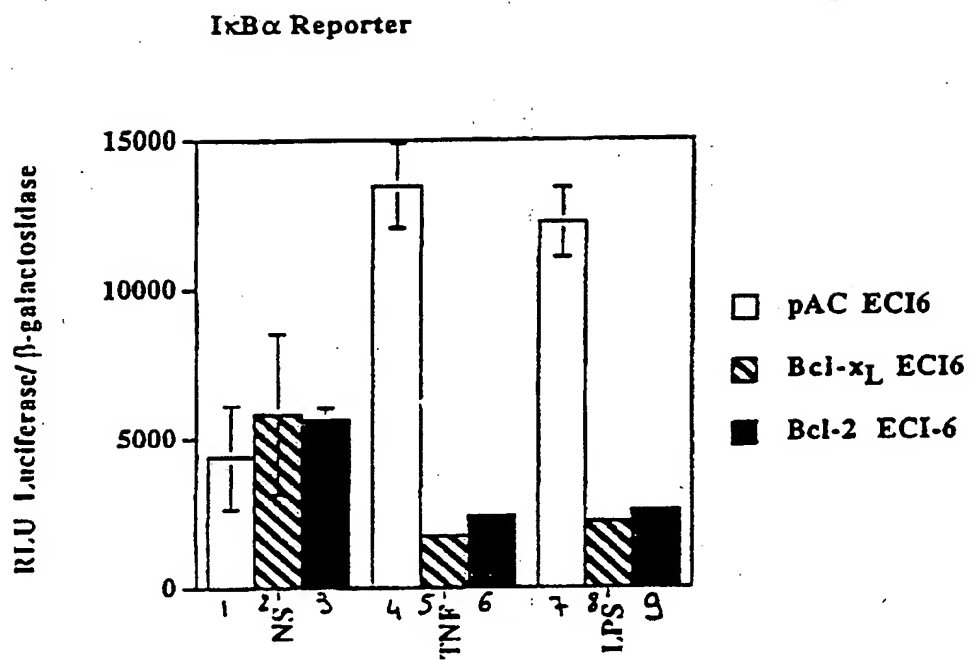
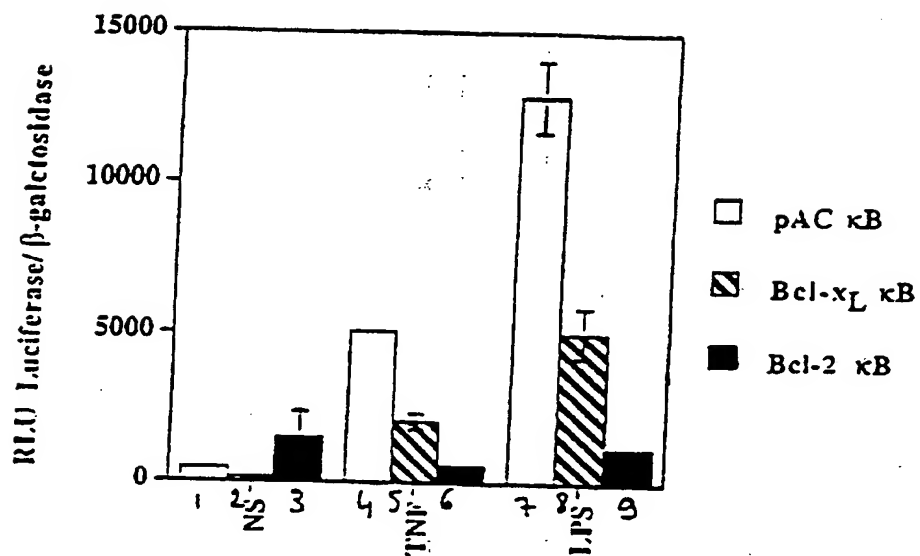
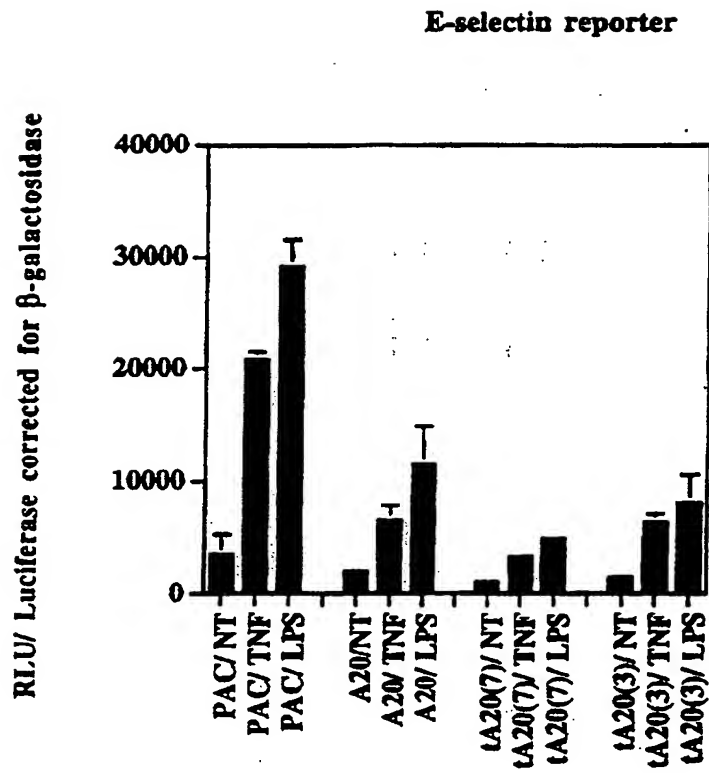


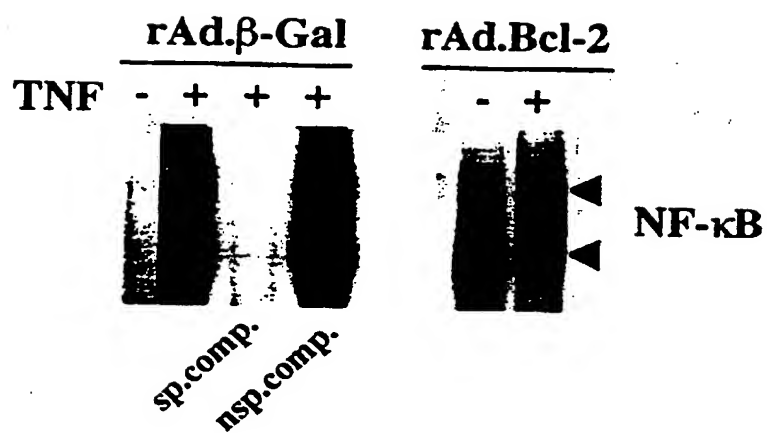
FIGURE 6A



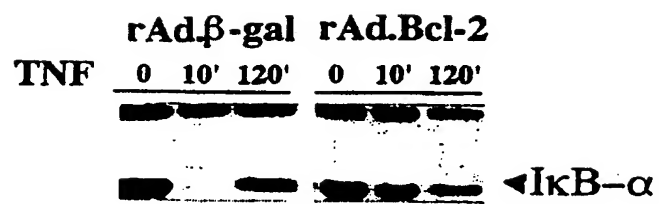


NF- $\kappa$ B Reporter









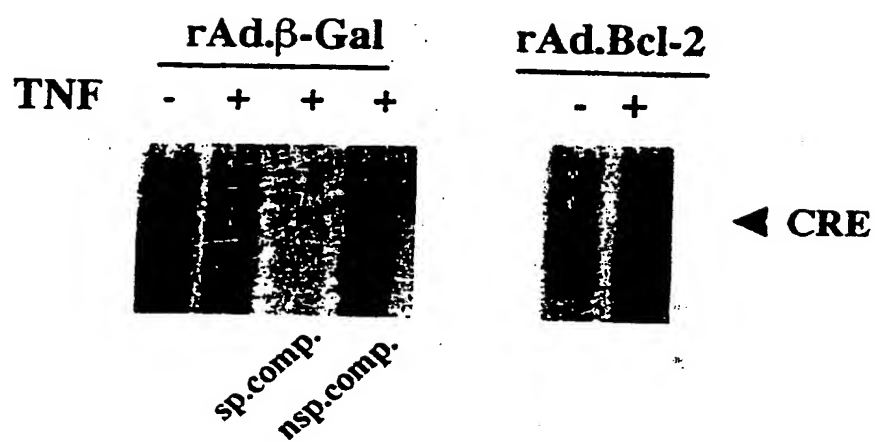


FIGURE 11 (Part A)

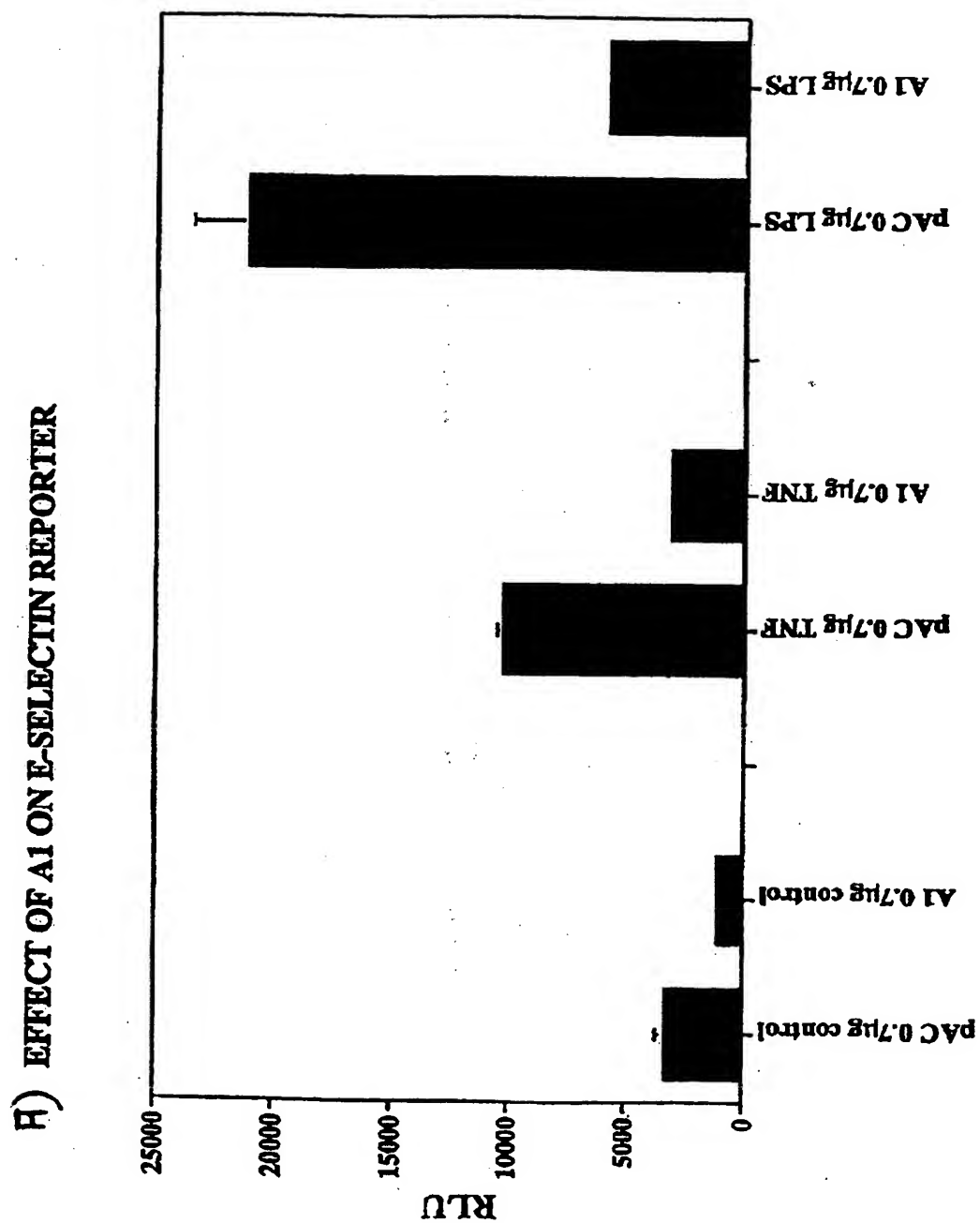
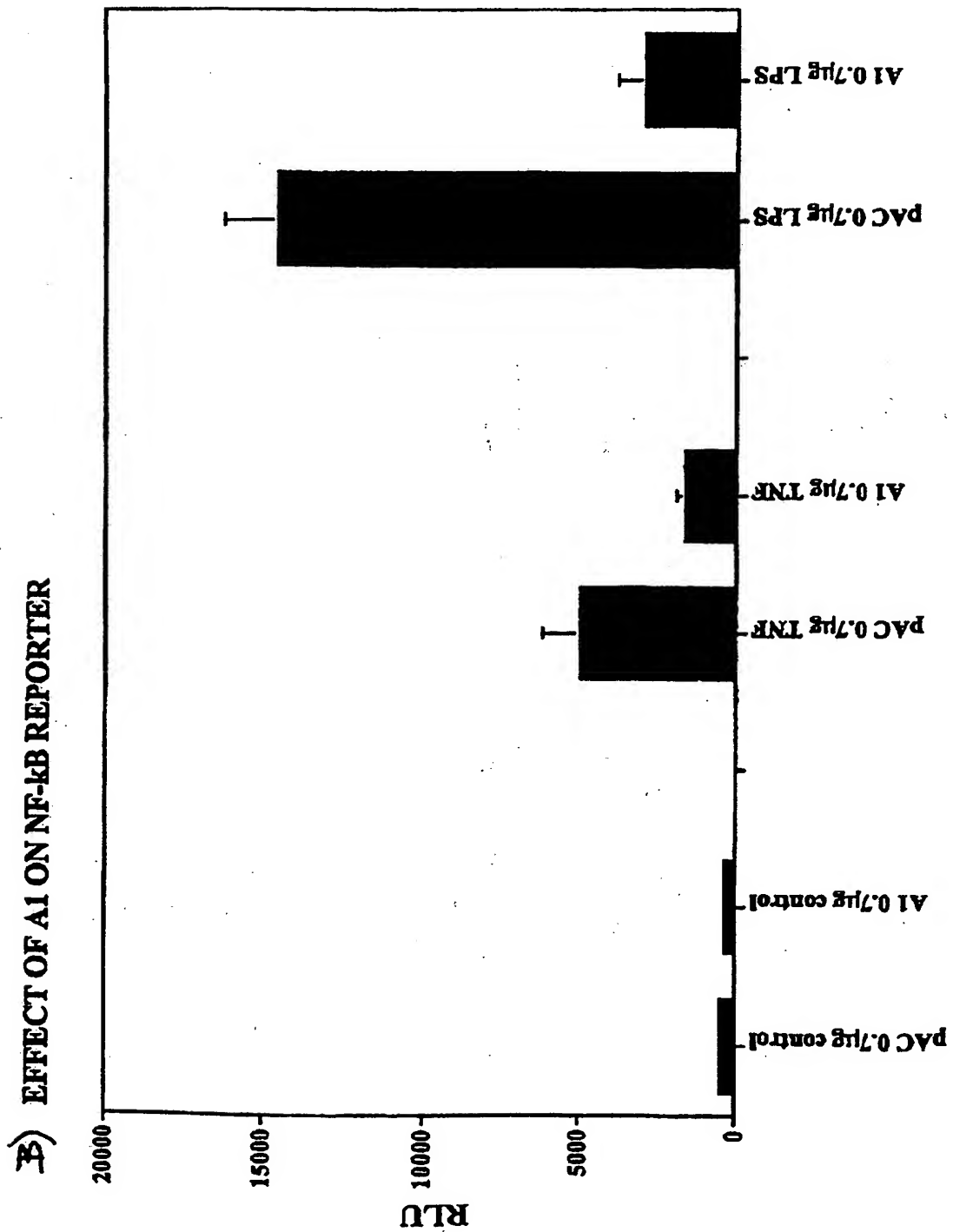
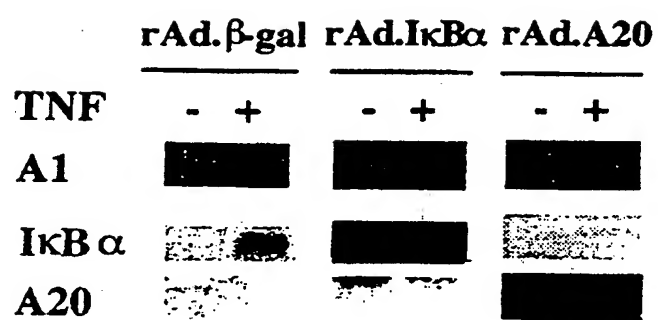


FIGURE 11 (Part B)





# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 97/00676

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K14/47 C12N5/10 C12N5/06 C07K14/82

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 10305 A (SANDOZ-ERFINDUNGEN VERWALTUNGSGESELLSCHAFT M.B.H.) 11 May 1994 see the whole document ---	1-11
X	JOURNAL OF IMMUNOLOGY, vol. 156, no. 3, 1 February 1996, BETHESDA, MD, US, pages 1166-1173, XP000676426 M. JAATTELA ET AL.: "A20 zinc finger protein inhibits TNF and Il-1 signaling" see the whole document ---	1-6
Y	WO 92 07573 A (SOMATIX THERAPY CORP ;HUGHES HOWARD MED INST; WHITEHEAD BIOMEDICAL) 14 May 1992 see page 11 see page 64, line 5-18 ---	1-8
-/--		

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search

3 July 1997

Date of mailing of the international search report

22.07.97

Name and mailing address of the ISA

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Fax (+31-70) 340-3016

Authorized officer

Mateo Rosell, A.M.

# INTERNATIONAL SEARCH REPORT

In. tional Application No  
PCT/EP 97/00676

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95 00642 A (ARCH DEVELOPMENT CORPORATION ; REGENT OF THE UNIVERSITY OF MICHIGAN) 5 January 1995 cited in the application see specially pages 10 and 11. see the whole document ---	1-8
A	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 214, no. 1, 1995, ORLANDO, FL, pages 212-223, XP000676429 C. FERRAN ET AL.: "Inhibition of NF-kappa-B by pirrolidine dithiocarbamate blocks endothelial cell activation" see the whole document ---	1-11
A	JOURNAL OF IMMUNOLOGY, vol. 154, 1995, BETHESDA, MD, US, pages 1699-1706, XP000673576 M. TEWARI ET AL.: "Lymphoid expression and regulation of A20, an inhibitor of programmed cell death" cited in the application see specially discussion see the whole document ---	1,3,6,8
A	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 267, no. 25, 1992, BETHESDA, MD, US, pages 17971-17976, XP000673587 A. KRIKOS ET AL.: "Transcriptional activation of the tumor necrosis factor alpha-inducible zinc finger protein, A20, is mediated by kappaB elements" cited in the application see the whole document ---	1-8
A	JOURNAL OF IMMUNOLOGY, vol. 155, 1995, BETHESDA, MD, US, pages 66-75, XP000673577 W. FANG ET AL.: "Bcl-XL rescues WEHI 231 B Lymphocytes from oxidant-mediated death following diverse apoptotic stimuli" cited in the application see the whole document ---	1,3,4,9
A	SOCIETY FOR NEUROSCIENCE ABSTRACTS, vol. 21, no. 2, 1995, BETHESDA, MD, US, page 1068 XP000197612 K-I. LIN ET AL.: "Bcl-2 suppresses apoptosis and activation of NF-kappa B induced by Sindbis virus infection" see abstract --- -/--	1-4,6

# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/EP 97/00676

## C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 93 19605 A (A.C. OCHOA ET AL. ) 14 October 1993 see abstract see page 36, line 6-26 ---	1-6,8
A	WO 92 20795 A (CETUS ONCOLOGY CORPORATION; UNIVERSITY OF NORTH CAROLINA ) 26 November 1992 see page 3 line 5-7 see the whole document ---	1,3,4,6,8
A	WO 93 20219 A (THE GOVERNMENT OF THE UNITED STATES) 14 October 1993 see abstract ---	1
P,X	BLOOD, vol. 87, no. 8, 15 April 1996, PHILADELPHIA, PA,US, pages 3089-3096, XP000197498 A. KARSAN ET AL.: "cloning of a human Bcl-2 homologue: inflammatory cytokines induce human A1 in cultured endothelial cells" cited in the application see specially discussion. ---	1,3,4,6,8
P,X	JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 30, 26 July 1996, BETHESDA,MD,US, pages 18068-18073, XP000677230 J.T. COOPER ET AL.: "A20 blocks endothelial cell activation through a NF-kappa-B-dependent mechanism" see the whole document ---	1-4,6
T	TRANSPLANTATION PROCEEDINGS, vol. 29, no. 1-2, 1997, STAMFORD, CT,US, pages 879-880, XP000197598 C. FERRAN ET AL.: "Adenovirus-mediated gene transfer of A20 renders endothelial cells resistant to activation: A means of evaluating the role of endothelial cell activation in xenograft rejection" see abstract ---	1-11
T	TANSPLANTATION PROCEEDINGS, vol. 29, no. 1-2, 1997, STAMFORD, CT, US, page 881 XP000197597 J.T. COOPER ET AL.: "A20 expression inhibits endothelial cell activation" see abstract -----	1-11



# INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP 97/00676

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 4, 5  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although these claims refer to a method of treatment of the human or animal body, the search was carried out and based on the alleged effects of the compound.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No  
PCT/EP 97/00676

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9410305 A	11-05-94	AU 5440494 A	24-05-94
		CA 2143489 A	11-05-94
		EP 0669977 A	06-09-95
		FI 952088 A	02-05-95
		JP 8502653 T	26-03-96
		NO 951628 A	03-07-95
		NZ 257539 A	27-08-96
WO 9207573 A	14-05-92	AT 147102 T	15-01-97
		AU 659824 B	01-06-95
		AU 1266692 A	26-05-92
		AU 656544 B	09-02-95
		AU 9017591 A	26-05-92
		CA 2095153 A	01-05-92
		CA 2095256 A	01-05-92
		DE 69123981 D	13-02-97
		DE 69123981 T	05-06-97
		EP 0568537 A	10-11-93
		EP 0556345 A	25-08-93
		ES 2096750 T	16-03-97
		JP 7503121 T	06-04-95
		JP 6503968 T	12-05-94
		WO 9207943 A	14-05-92
WO 9500642 A	05-01-95	AU 7177394 A	17-01-95
		CA 2165969 A	05-01-95
		EP 0706568 A	17-04-96
		JP 9502603 T	18-03-97
WO 9319605 A	14-10-93	US 5556763 A	17-09-96
		US 5296353 A	22-03-94
		US 5583002 A	10-12-96
		AU 3939593 A	08-11-93
		CA 2133759 A	14-10-93
		EP 0634896 A	25-01-95
		JP 8504170 T	07-05-96
		ZA 9302434 A	23-11-93
WO 9220795 A	26-11-92	AU 2140592 A	30-12-92
		AU 7062196 A	06-02-97

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No  
PCT/EP 97/00676

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9220795 A		CA 2102704 A EP 0584238 A JP 6508029 T	18-11-92 02-03-94 14-09-94
WO 9320219 A	14-10-93	AU 4045193 A	08-11-93

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